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Haptens, bioconjugates, and antibodies for penthiopyrad immunosensing†

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Haptens, bioconjugates, and antibodies for highly sensitive immunochemical analysis of the new-generation fungicide penthiopyrad are described. Two haptens with equivalent carboxylated linkers were prepared, and the purified active esters were efficiently coupled to proteins. The results revealed slightly different antibody-eliciting capacities for the two synthetic derivatives. All of the produced antibodies were specific for penthiopyrad, and showed affinity values in the nanomolar range.

Penthiopyrad (Fig. 1) is a new-generation pyrazole carboxamide fungicide that interferes with fungal cell respiration in complex II of the cytochrome system by inhibiting the succinate dehydrogenase activity (Group 7).[1] Penthiopyrad is active against many diseases caused by ascomycetes, deuteromycetes, and basidiomycetes,[2] and due to its unique mode and site of action, it has shown great potential as complementary active principle in order to fight against fungi resistance to other frequently used fungicides such as strobilurins and sterol biosynthesis inhibitors.[3,4] Penthiopyrad was first commercially registered in the United States in 2012 and it has been recently approved by the European Commission.[5]

Nowadays, antibody-based technologies constitute valuable complementary and/or alternative methods for chemical residue and contaminant analysis.[6] In order to achieve good acceptance and widespread implementation of immunosensors in quality control laboratories, appropriate reagents are required and alternative analytical approaches are in demand.

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† Electronic supplementary information (ESI) available: General methods and instruments, experimental details of hapten synthesis (Schemes S1–S3), spectrometric characterization data, hapten activation and conjugation details, MALDI spectra of conjugates, antibody generation, competitive ELISA procedures, standard curves (Fig. S1), and ¹H NMR spectra of haptens PPa and PPb. See DOI: 10.1039/c4an00828f

Therefore, research projects are needed aiming to increase the scientific knowledge about the processes and factors influencing the analytical properties of antibodies and the development of sensitive procedures.

For small chemicals, such as penthiopyrad, functionalized derivatives of the target molecule containing a linker arm and an activatable chemical group for conjugation to the carrier are usually essential.[7–9] The main determinant groups of the target compound must be displayed unmodified in the immunizing conjugate. This rule can generally be achieved by introducing a carbohydrate aliphatic linear spacer at the appropriate site of the analyte.[10] Moreover, the optimum hapten design will benefit not only antibody selectivity and affinity but also assay performance.[11] The aim of this study was to investigate different derivatives of penthiopyrad with alternative linker tethering sites and to generate protein conjugates as well as selective and high-affinity antibodies in order to develop sensitive immunoassays for the rapid analysis of this fungicide.
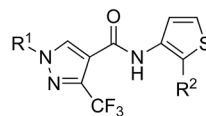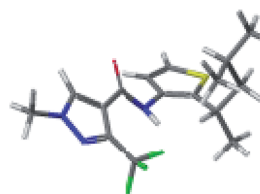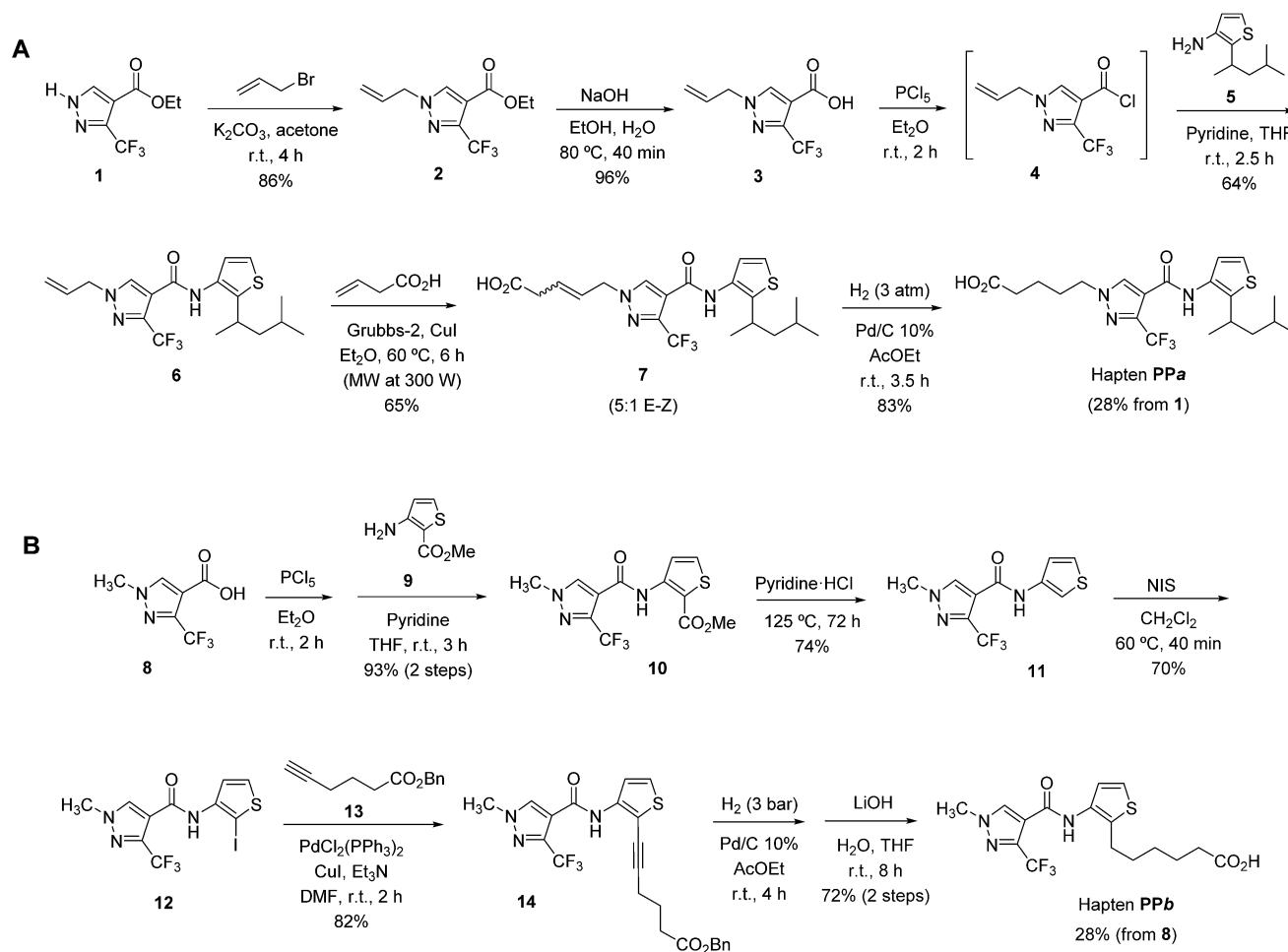

Penthiopyrad: R¹ = CH₃; R² = CH(Me)CH₂CHMe₂
Hapten PPa: R¹ = (CH₂)₄CO₂H; R² = CH(Me)CH₂CHMe₂
Hapten PPb: R¹ = CH₃; R² = (CH₂)₅CO₂H

Fig. 1 Chemical structures of penthiopyrad and the two synthetic haptens. Global minimum energy conformer of penthiopyrad calculated by CACHe/CONFLEX/MM3.

Following those principles, two penthiopyrad haptens – hapten PP*a* and hapten PP*b* (Fig. 1) – were designed mimicking as much as possible the structural and electronic properties of the parent analyte. In each of them, the spacer arm was located at opposite positions of the penthiopyrad framework in order to favour alternative display orientations to the immune system. Hapten PP*a* came from the formal replacement of the pyrazole *N*-methyl group by a C5 carboxylated aliphatic linear chain, which presumably only implied a minimum modification of the electronic and steric properties of the parent compound, whereas in hapten PP*b* a similar linear spacer arm replaced the branched 4-methylpentan-2-yl substituent at the 2-position of the thiophene ring. In the latter case, there was a greater alteration of the penthiopyrad framework, especially from the steric point of view, but it only affected the proximal part of the hapten in the immunogenic conjugate.

Since none of the two designed haptens can be prepared by chemical transformations from penthiopyrad or other structurally related accessible compounds, it was necessary to develop a total synthesis of both molecules from commercially available materials. The synthetic sequences developed for the preparation of haptens PP*a* and PP*b* are shown in Schemes 1A and B, respectively. Every intermediate of the synthesis as well

as the final haptens were purified and fully characterized by spectroscopic methods (for details see the ESI, Schemes S1–S3†). A brief discussion of each of the synthetic procedures is also given in the ESI.† The synthesis of hapten PP*a* was accomplished *via* a six step sequence in 28% overall yield, while the synthesis of hapten PP*b* required seven reaction steps with a calculated overall yield of *ca.* 30%. In both cases, the overall yields can be considered very satisfactory due to the number and type of reactions that were involved, showing the effectiveness of the developed synthetic sequences.

Carboxyl-functionalized haptens were activated using N,N'-disuccinimidyl carbonate in acetonitrile following an adapted strategy that was optimized in previous studies for other compounds,[12] which allowed a straightforward purification and characterization of the resulting active *N*-hydroxysuccinimidyl esters. Immunizing conjugates with BSA and assay conjugates with OVA and HRP were readily prepared by adding the corresponding pure active ester of the hapten over buffered protein solutions. Data for hapten activation and conjugation are given in the ESI.† Finally, covalent hapten-to-protein complexes were purified by size exclusion chromatography. This step-by-step approach avoided the formation of undesired secondary products during protein coupling, and therefore the same strategy

Scheme 1 Schematic representation of the synthesis of hapten PP*a* (A) and hapten PP*b* (B).

could be employed both for immunizing and assay conjugate preparation, even for polyclonal antibody-based immunoassays. Moreover, since the conjugation reaction was more efficient, lower amounts of hapten were required. The number of conjugated hapten molecules was determined by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF/MS). The hapten-to-protein molar ratios (MR) obtained for the immunogens were 19.2 and 15.1 for conjugates BSA–PP*a* and BSA–PP*b*, respectively (Fig. S1 in the ESI†). The MRs for assay conjugates were 2.7 and 3.2 for conjugates OVA–PP*a* and OVA–PP*b*, respectively, and 2.9 and 2.2 for conjugates HRP–PP*a* and HRP–PP*b*, respectively (see Fig. S2 and S3 in the ESI†).

Animal manipulation was performed in compliance with the laws and guidelines of the Spanish Ministry of Agriculture, Food, and Environment. Antibodies were generated in rabbits by immunization with 0.3 mg of BSA–hapten conjugate using Freund's adjuvants. Partial antibody purification was performed by salting out twice. Further details are provided in the ESI.† Antibody-coated direct cELISAs and conjugate-coated indirect cELISAs were performed using homologous and heterologous conjugates as described in the ESI.† Mean absorbance values were plotted *versus* the logarithm of the analyte concentration, and assay sensitivity to penthiopyrad was estimated as the analyte concentration reducing 50% (IC_{50}) the maximum absorbance (A_{max}).

From each penthiopyrad hapten, two polyclonal antibodies were obtained; namely, antibodies PP*a*#1 and PP*a*#2 from hapten PP*a*, and antibodies PP*b*#1 and PP*b*#2 from hapten PP*b*. Those antibodies were evaluated by checkerboard cELISA with the two hapten conjugates. Eight-point standard curves were prepared by a 10-fold serial dilution, including a blank point. A 10 mM penthiopyrad stock solution in *N*,*N*-dimethylformamide was used to prepare the first point of the standard curve. One penthiopyrad standard curve was run in every microplate column, and different antibody or conjugate concentrations were assayed in the same plate. For the direct format, three different antibody dilutions (1/3000, 1/10 000, and 1/30 000) and four enzyme tracer concentrations (3, 10, 30, and 100 ng mL^{-1}) were assayed in the same plate. For indirect competitive assays, plates were coated with the 100 or 1000 ng mL^{-1} OVA conjugate, and diverse antibody dilutions were assayed from 1/3000 to 1/300 000 in one plate. Thus, a series of twelve inhibition curves was retrieved from every plate; each curve corresponding to a particular antibody and assay conjugate combination at a specific concentration.

A sufficient assay signal (over 0.8) at a zero dose of analyte was achieved with every antibody/conjugate combination in both assay formats, with the only exception of antibody PP*a*#1 which did not bind the heterologous enzyme tracer HRP–PP*b* (Table 1). All assays showed negligible background signals, in spite of using the same coupling procedure for immunizing and assay conjugate preparation. The observed IC_{50} values were mostly in the low nanomolar range demonstrating the suitability of the prepared haptens and immunogens. The affinities for penthiopyrad of antibodies that were generated from hapten PP*a* were somewhat higher than those of the antibodies obtained from

hapten PP*b*, indicating that hapten PP*a* was a better mimic of the target analyte as discussed previously for hapten design.

The IC_{50} values of PP*a*-type antibodies could be lowered by using the heterologous conjugates with hapten PP*b*. Such improvement was also observed with PP*b*-type antibodies in the indirect assay format, but not in the direct format (Table 1). These results show the importance of preparing different functionalized haptens, not only for high affinity antibody production but also for sensitive assay development. Linker site heterologies are adequate for assay sensitivity improvement; however, for particular antibodies and assay formats, this could be a too drastic modification making it difficult to achieve sufficient signal. In both cELISAs, the best results were obtained with antibody PP*a*#2 combined with the heterologous conjugate; IC_{50} values of 1–2 nM with OVA–PP*b* or HRP–PP*b*, according to the assay format. The standard curves of the most sensitive immunoreagent combination by direct and indirect cELISA can be seen in Fig. S4 of the ESI.†

Antibody selectivity was studied by direct cELISA. Cross-reactivity (%) with other fungicides was determined from the quotient between the IC_{50} values for penthiopyrad and for the corresponding assayed compound, both in molar concentration units. As competitors, different pyrazole bioactive principles (pyraclostrobin, fluxapyroxad, fluopyram, and fluopicolide) and other common fungicides (azoxystrobin, boscalid, pyrimethanil, cyprodinil, fenhexamid, fludioxonil, and tebuconazole) were evaluated. For PP*a*-type antibodies, no inhibition was found at a concentration of 10 μM with any of the studied compounds. Regarding the two PP*b*-type antibodies, a moderate inhibition was observed only with fluxapyroxad, which contains a similar pyrazole carboxamide group to that of penthiopyrad – the calculated cross-reactivity was below 0.5% for both PP*b*-type antibodies. This result is in accordance with Landsteiner's principle,[13] stating that antibody specificity is predominantly directed towards the hapten moieties positioned opposite to the linker tethering site. In fact, the spacer arm location in hapten PP*b* was distal to the pyrazole ring.

In conclusion, two haptens were designed with equivalent carboxylated linkers at opposite tethering sites of the target molecule. Haptens PP*a* and PP*b* were achieved in six and seven steps, respectively, by the total synthesis of the functionalized derivative. Purified active esters of the haptens were employed for efficiently preparing both the immunizing and the assay conjugates. High-affinity polyclonal antibodies to penthiopyrad were generated from both haptens. To the best of our knowledge, these are the first reported haptens, bioconjugates, and antibodies to this modern fungicide. Antibodies from hapten PP*a* displayed a moderately higher affinity and selectivity than those obtained from hapten PP*b*. The replacement of the branched aliphatic substituent at the thiophene ring (Fig. 1) by the linear hydrocarbon spacer arm (hapten PP*b*) did not hamper the generation of antibodies with IC_{50} values in the nanomolar range – probably because such minor alteration was located at a proximal position – though it was shown to be a slight hindrance to antibody affinity. Finally, antibody PP*a*#2 was identified as a promising candidate for sensitive immunosensor development and sample analysis.

Table 1 Checkerboard assays in two cELISA formats ($n = 3$)

Direct cELISA

	Tracer conjugate							
	HRP–PPa				HRP–PPb			
Ab	[Ab][a]	[T][b]	A_{max}	IC$_{50}$ (nM)	[Ab]	[T]	A_{max}	IC$_{50}$ (nM)
PPa#1	10	10	1.22 ± 0.16	4.39 ± 0.21	3	300	ns[c]	—
PPa#2	10	10	1.24 ± 0.25	4.73 ± 0.62	3	300	0.99 ± 0.14	1.22 ± 0.16
PPb#1	3	100	0.83 ± 0.14	13.79 ± 4.71	3	100	1.09 ± 0.23	7.05 ± 3.07
PPb#2	3	100	0.80 ± 0.14	6.47 ± 1.08	10	30	0.88 ± 0.24	3.14 ± 0.99

Indirect cELISA

	Coating conjugate							
	OVA–PPa				OVA–PPb			
Ab	[Ab]	[C][d]	A_{max}	IC$_{50}$ (nM)	[Ab]	[C]	A_{max}	IC$_{50}$ (nM)
PPa#1	100	100	0.94 ± 0.12	3.25 ± 0.90	30	100	1.24 ± 0.09	3.29 ± 0.96
PPa#2	100	100	0.95 ± 0.13	2.23 ± 0.36	30	100	1.47 ± 0.45	1.83 ± 0.14
PPb#1	3	100	1.30 ± 0.03	6.06 ± 1.06	30	100	0.90 ± 0.14	22.37 ± 1.67
PPb#2	10	100	1.27 ± 0.10	9.27 ± 3.97	30	100	1.88 ± 0.32	34.84 ± 4.10

[a] Antibody dilution ($\times 10^3$). [b] Tracer concentration in ng mL^{-1}. [c] Signal was lower than 0.8. [d] Conjugate concentration in ng mL^{-1}.

Acknowledgements

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