Hapten Design and Antibody Generation for Immunoanalysis of Spirotetramat and Spirotetramat-enol

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Supporting Information

ABSTRACT: Spirotetramat—a tetramic acid insecticide—is rapidly metabolized or degraded to give spirotetramat-enol; so, common residue definitions include the sum of both compounds. In the present study, two spirotetramat-functionalized derivatives (haptens) have been designed to generate immunoreagents to these molecules for rapid immunochemical analysis. Haptens have been synthesized with alternative linker tethering sites and, for the first time, high-affinity antibodies have been generated with different specificities to these active principles. Two sensitive assays have been developed using the same antibody in different formats, and by using linker-site heterologous haptens, the selectivity of the final immunoassay could be improved. A generic immunoassay with sensitivity similar to spirotetramat and spirotetramat-enol and a specific assay of spirotetramat-enol have been developed. The described antibody and bioconjugates showed great potential for sensitive immunoSENSOR development and analysis of this complex analyte.

1. INTRODUCTION

Spirotetramat (SP) is the ISO common name for cis-4-(ethoxycarbonyloxy)-8-methoxy-3-(2,5-xylyl)-1-azaspiro[4.5]-dec-3-en-2-one (IUPAC). This is a new-generation biocide that was discovered as a derivative of the natural antibiotic thiolactomycin. In 2013, SP was included in Annex I to Regulation (EC) number 1107/2009 of the European Parliament and of the Council concerning the placing of plant protection products on the market. It belongs to the tetramic acid family, and it is structurally characterized by a spirocyclic group containing a dimethylphenyl group and an ethyl carbonate group as substituents (Figure 1). SP shows a new mode of action that consists of inhibiting the acetyl-CoA carboxylase activity, thus blocking the biosynthesis of lipids in a variety of target insect pests. Upon absorption by the organism, SP is transformed into a more polar enol derivative (SP-enol) by the hydrolytic cleavage of the ethyl carbonate group (Figure 1), thus facilitating its translocation. Moreover, SP is a labile compound that degrades at ambient temperature and particularly under alkaline conditions—the half-life at pH 9 and 25 °C is 7.6 h—SP-enol being the major degradation product. Further metabolism or degradation of SP-enol can give rise to several supplementary and frequently minor derivatives, such as spirotetramat-enol glucoside (SP-glc), spirotetramat-ketohydroxy (SP-keto), spirotetramat-monohydroxy (SP-mono), and spirotetramat-desmethyl-enol. SP shows moderate-to-low acute toxicity in mammals—LC50 > 4.183 mg/L by inhalation and LD50 > 2000 mg/kg via oral and dermal routes in rats, but it has been demonstrated to be rather toxic to aquatic organisms.

Analysis of SP is commonly carried out by high-performance liquid chromatography with photometric or mass spectrometry detection, frequently after quick, easy, cheap, effective, rugged, and safe extraction of samples. However, the determination of SP residues in environmental and food samples can be quite complex because of the diversity of SP metabolites or degradates that can be present in the sample. For risk assessment studies, both the parent compound and the metabolites are taken into consideration, whereas a common definition of SP residues for enforcement or compliance with
the maximum residue limits in plant commodities is the sum of SP and SP-enol, expressed as SP concentration, because both compounds mostly constitute the bulk of the residues.\textsuperscript{11,12}

Antibody-based analytical techniques are nowadays employed as complementary strategies for small chemical contaminants and residue monitoring. The competitive enzyme-linked immunosorbent assay (eELISA) is probably the most extended immunochemical method because of its large sample throughput and its ability to provide quantitative results.\textsuperscript{13} However, high-quality immunoreagents are required for attaining these goals. Covalent bioconjugates of the target compound should be primarily obtained and employed as immunogens to raise antibodies. Moreover, the bioconjugates are necessary to develop competitive immunoassays. To our knowledge, the preparation of bioconjugates and the generation of antibodies to SP have not been reported previously, and consequently no bioanalytical methods have been described so far for this complex analyte. The aim of our study was to raise valuable immunoreagents and to develop sensitive and rapid immunochemical techniques for the analysis of SP and SP-enol.

2. RESULTS AND DISCUSSION

2.1. Hapten Design and Synthesis. Two functionalized derivatives of SP (hapten) with alternative linker tethering sites were designed and synthesized, namely SP\textsubscript{c} and SP\textsubscript{o} (Figure 1), to generate antibodies with different specificities. These haptenes incorporated, through two of the oxygenated functions thereof, a carboxylated hydrocarbon chain that served as a spacer arm for its conjugation to the carrier proteins. In both cases, the SP framework was fully maintained, and the introduction of the spacer arm through a C–O bond ensured minimal modifications of the SP electronic properties, so that they could be deemed as adequate mimics of the analyte’s parent structure.

The synthesis of hapten SP\textsubscript{c} (Figure 1) started from the metabolite SP-enol (1), which is commercially available and also readily obtainable from SP by the basic hydrolysis of the enol-carbonate moiety at the C-2 position.\textsuperscript{1} The incorporation of the spacer arm at the enolic oxygen atom was carried out in a three-step sequence starting with the formation of allyl carbonate 2, via the reaction of SP-enol with allyl chloroformate, followed by a microwave-assisted cross-metathesis reaction with 4-pentenoic acid to form a ca. 9:1 trans/cis mixture of the corresponding olefin 3. Finally, double-bond hydrogenation under homogeneous conditions using Wilkinson’s catalyst completed the synthesis of hapten SP\textsubscript{c}. Thus, the synthesis of hapten SP\textsubscript{c} was accomplished from 1 via three steps in a 49% overall yield. The structure of the hapten and those of the intermediates were confirmed by infrared (IR) spectroscopy, proton nuclear magnetic resonance (\textsuperscript{1}H NMR), carbon (\textsuperscript{13}C) NMR, and high-resolution mass spectrometry (HRMS) (data can be found in section 4.2).

The preparation of hapten SP\textsubscript{o} (Figure 1) was readily undertaken from demethylated SP (5). This compound can be directly obtained from SP itself,\textsuperscript{14} although for this work we prepared it from ketone 4, which was in turn obtained through a synthetische route similar to the one previously described.\textsuperscript{15} Thus, the ketone carbonyl group of 4 was stereoselectively reduced under the Luche conditions to the equatorial hydroxyl group from which the spacer chain was introduced through an acylation reaction with glutaric anhydride and catalytic zinc perchlorate hexahydrate under microwave irradiation.\textsuperscript{16} Hapten SP\textsubscript{o} was thus obtained with an overall yield for the two steps of about 44%. The chemical structures of the hapten and the intermediates were verified by IR spectroscopy, \textsuperscript{1}H NMR, \textsuperscript{13}C NMR, and HRMS (extended data are reported in section 4.3).

2.2. Preparation of Bioconjugates. Prior to coupling with proteins, haptenes SP\textsubscript{c} and SP\textsubscript{o} were activated through the formation of active esters. This activation procedure and the subsequent chromatographic purification led to the corresponding N-hydroxysuccinimidyld esters—SP\textsubscript{c}-NHS and SP\textsubscript{o}-NHS esters (Figure 1)—in practically pure form, thus allowing a precise control of the coupling conditions and avoiding undesired secondary reactions.

Hapten SP\textsubscript{o} was coupled to bovine serum albumin (BSA) at basic pH to better promote the formation of amide bonds...
between the carboxyl group of the hapten and the amine groups of the protein. However, under these conditions, we cannot rule out the fact that partial hydrolysis of the ethyl carbonate group of the analyte might take place; if so, the immunogen would be a mixture between SP and SP-enol, which, far from being a drawback, may be convenient because both substances are included in the SP residue definition. A hapten-to-protein molar ratio (MR) of 13 was obtained, which is an optimum value for an immunizing conjugate (Figure 2A).

On the other hand, hapten SP\(_c\) was coupled to BSA at neutral pH to avoid the hydrolysis of the carbonate group which, in this case, would have caused the loss of the SP backbone, thus forming a bioconjugate with probably only the spacer arm. A rather low hapten-to-protein MR of 8 was attained in this conjugate (Figure 2A). The lower hapten-to-protein MR of the SP\(_c\) conjugate, as compared with that of hapten SP\(_o\), was most probably due to the greater coupling efficiency of the active ester method at basic pH, as already mentioned. Assay conjugates based on ovalbumin (OVA) and horseradish peroxidase (HRP) were also prepared for both haptens at neutral pH and, in terms of applicability, good MRs (3−4 for OVA and 1 for HRP) were obtained (Figure 2).

### 2.3. Antibody Generation and Characterization.

Two antisera were obtained from each BSA conjugate, which were...
IC50 values were lower for SP-enol than for SP. This result might have been generated. Concerning SPo-type antibodies, high Amax values were obtained at very low homologous coating conjugate concentrations, regardless of the coating pH. More significantly, both SP and SP-enol inhibited the immunochemical reaction between the antibodies SPo#1 and SPo#2 and the coating conjugate, even though the IC50 values were lower for SP-enol than for SP. This result could be explained by a partial loss of the ethyl carbonate group in the SP0 hapten during conjugation and immunization; so, in practice, animals would have been immunized with a mixture of a conjugate of SP and an in vivo-formed conjugate of SP-enol.

The obtained antibodies were also evaluated by the antibody-coated direct and the capture antibody direct cELISA formats, and similar results were obtained among each other. Not surprisingly, binding between SPc-type antibodies and HRP–SPc tracer was not observed (Table 2). On the contrary, binding to the homologous enzyme tracer (HRP–SPo) was found with both SPo-type antibodies in the two formats, and, more importantly, inhibition was observed with SP and SP-enol. Interestingly, both antibodies showed a much higher affinity to SP-enol than to SP, probably because of the partial hydrolysis of hapten SPo in the immunizing conjugate, as already mentioned. The lowest IC50 value for SP-enol (1.5 nM) was found with the antibody SPo#1 in the capture format.

### 2.4. Immunoassay Development

The performance of the four antibodies in combination with the heterologous conjugate—in which the hapten is different to that of the immunizing conjugate—was assessed in the three studied cELISA formats. Unfortunately, none of the antibodies recognized the heterologous enzyme tracer; so, no signal was observed with the direct and the capture assay formats. The situation was quite similar for the indirect format, in which the antibodies poorly recognized the heterologous coating conjugates (Table 3); so, no feasible assays could be developed. Higher MR values of the OVA–SPo conjugate (MR = 15) did not improve the Amax values. The only exception to this general finding was antibody SPo#1; in plates coated at neutral pH with the OVA–SPc heterologous conjugate, an inhibition curve with an Amax value around 1.0 could be obtained. More notably, the observed IC50 values with the immunoreagent combination were 100-fold (9.47 nM) and 30-fold (3.60 nM) lower for SP and SP-enol, respectively, than the values obtained with the homologous assay. Accordingly, further work was carried out only with antibody SPo#1, either combined with the heterologous coating conjugate or not.

Table 2. Checkerboard Titration of Antibodies and Bioconjugates by Direct and Capture Antibody Direct cELISA (n = 3)

| assay format | enzyme tracer | [T]a | pAb | antibody titer | Amax | slope | IC50 (nM) SP | IC50 (nM) SP-enol |
|--------------|---------------|------|-----|----------------|------|--------|--------------|-----------------|
| direct       | HRP–SPc       | 300  | SPC#1 | 3000           | n.s. | n.s.   |              |                 |
| capture      | HRP–SPc       | 30   | SPC#1 | 10 000         | 0.69 | 0.74   | 165          | 10.8            |
|              | HRP–SPc       | 300  | SPC#2 | 3000           | 1.02 | 0.78   | 233          | 19.6            |
|              | HRP–SPo       | 100  | SPO#1 | 30 000         | 1.07 | 0.71   | 113          | 1.5             |

“Tracer concentrations are in ng/mL. bNo signal was observed.

Table 3. Checkerboard Titration of Antibodies and Bioconjugates by Indirect cELISA Using the Heterologous Coating Conjugate (n = 3)

| coating conjugate | coating pH | [C]a | pAb | antibody titer | Amax | slope | IC50 (nM) SP | IC50 (nM) SP-enol |
|-------------------|------------|------|-----|----------------|------|--------|--------------|-----------------|
| OVA–SPo           | 9.6        | 300  | SPO#1 | 3000           | 0.33 | 0.77   | 4.10         |                 |
|                   |            | 300  | SPO#2 | 3000           | 0.49 | 0.75   | 7.89         |                 |
|                   | 7.4        | 300  | SPO#1 | 3000           | 0.24 | 0.89   | 5.32         |                 |
|                   |            | 300  | SPO#2 | 3000           | 0.31 | 0.79   | 96.6         |                 |
| OVA–SPc           | 9.6        | 300  | SPC#1 | 3000           | 0.32 | 0.66   | 3.27         | 3.58            |
|                   |            | 300  | SPC#2 | 3000           | 0.10 | 0.52   | 12.0         | 1.71            |
|                   | 7.4        | 300  | SPC#1 | 3000           | 1.02 | 0.94   | 9.47         | 3.60            |
|                   |            | 300  | SPC#2 | 3000           | 0.28 | 0.63   | 9.96         | 20.1            |

“Conjugate concentrations are in ng/mL. bNo inhibition.
conjugate (OVA–SPc) in the indirect format or together with the homologous tracer (HRP–SPo) in the capture antibody direct format.

The selectivity of both immunoassays to the four main metabolites of SP, that is, SP-enol, SP-glc, SP-keto, and SP-mono, was assessed. The homologous capture assay was pretty specific of SP-enol, as previously described. The cross-reactivity (CR) values, referred to SP-enol, were 1.3% for SP, 2.2% for SP-glc, and almost negligible for SP-keto and SP-mono. However, the heterologous indirect assay exhibited a different recognition pattern, with SP and SP-enol being recognized to a closer extent, whereas the other compounds were again much less recognized. The CR values were 38 and 100% for SP and SP-enol, respectively, 2.3% for SP-glc, and lower than 0.5% for the rest of the metabolites (Table 4).

Table 4. Immunoassay Cross-Reactivity (%,

| Analyte      | Indirect | Capture |
|--------------|----------|---------|
| SP           | 38       | 1.3     |
| SP-enol      | 100      | 100     |
| SP-glc       | 2.3      | 2.2     |
| SP-keto      | 0.2      | <0.1    |
| SP-mono      | <0.1     | 0.1     |

Interestingly, the two selected immunoassays employed the same antibody but different assay conjugates. The heterology introduced by hapten SPc in the indirect assay made the IC₅₀ value for SP closer to that of SP-enol, thus resulting in a more generic assay for both compounds. The presence of the carbonate group in SPc probably selects the fraction of antibody molecules that bind the nonhydrolyzed hapten, thus increasing the apparent affinity to SP. The standard curves for SP and SP-enol in the two optimized immunoassays are shown in Figure 3. The logarithm of the odds (LOD) values, calculated as IC₁₀ of the standard curve, were 0.5 and 0.6 nM for SP and SP-enol, respectively, in the indirect assay. On the other hand, the LOD values for the capture assay were almost 100 times higher for SP than for SP-enol (5 and 0.06 nM, respectively). These values are in the same range of those of the reference chromatographic methods,¹⁰,¹⁷,¹⁸ and they suggest the great potential of the developed immunoassays for SP and SP-enol residue determination.

3. CONCLUSIONS

Two functionalized derivatives of SP have been synthesized with equivalent linkers located at different sites of the molecule. The lability of the carbonate group of the molecule was the determinant for the generation of antibodies to SP and SP-enol. A cELISA specific of SP-enol was established with the capture antibody format using the antibody SPoFF and the homologous enzyme tracer. Moreover, a generic cELISA for SP and SP-enol was characterized in the conjugate-coated indirect format using the same antibody but combined with a heterologous conjugate. Highly sensitive immunoassays, with the LOD values in the low nanomolar scale, have been presented as a proof of principle for the immunoanalysis of this complex chemical residue.

4. MATERIALS AND METHODS

4.1. Reagents and Instruments. SP, spirotetramat-enol, and the rest of the metabolites were of Pestanal grade and were purchased from Merck (Madrid, Spain). Further information about the reagents and equipment is provided in the Supporting Information.

4.2. Synthesis of Hapten SPc. This hapten was prepared in three synthetic steps as schematized in Figure 1. The ¹H NMR spectrum of hapten SPc is provided in the Supporting Information.

Figure 3. Standard curves of the selected immunoassays. The A₅₆₅ value was around 1.0, and the background signal was always near zero. The values are the average of three independent experiments.
4.3. Preparation of Hapten SPo. To prepare this hapten (Figure 1), ketone 4 was obtained as described by Zhao et al. 15

The 1H NMR spectrum of hapten SPo is provided in the Supporting Information.

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4.3.1. Preparation of 3-(2,5-Dimethylphenyl)-8-hydroxy-2-oxo-1-azaspiro[4.5]dec-3-enediyloxy-carbonyl-oxoxyhexanoic Acid (Hapten SPa). A solution of acid 3 (59 mg, 0.129 mmol) and Wilkinson catalyst (10 mg, 0.011 mmol, 8%) in anhydrous triethylamine (2.5 ml) was evacuated and purged under an atmosphere of hydrogen gas. The hydrogen pressure was regulated to 55 psi and the reaction mixture was stirred at room temperature overnight. Then, the solvents were removed under vacuum, and the residue was purified by chromatography, using CHCl3/MeOH 99:1 as the eluent, to afford acid 3 (85 mg, 85%) as an approximately 8:2 mixture of trans/cis double-bond isomers. IS νmax (cm⁻¹): 3273, 2935, 1776, 1693, 1443, 1204, 1099, 968, 728; 1H NMR (CD3OD, 300 MHz), only the signals of the trans isomer are given: δ 7.90 (1H, s, NH), 7.12 (1H, d, J = 7.8 Hz, H-3 Ph), 7.07 (1H, dd, J = 7.8, 1.5 Hz, H-4 Ph), 6.91 (1H, d, J = 1.5 Hz, H-6 Ph), 5.78–5.61 (1H, m, H-4), 5.34 (1H, dt, J = 15.6, 6.4 Hz, 1H, s, OMe), 3.39 (3H, s, OMe), 3.00 (1H, m overlapped with the solvent signal, H-8′), 2.35–2.25 (4H, H-2, H-3), 2.29 and 2.18 (3H each; each s, 2H, Me-Ph), 2.18–2.10 (2H, 2H, Me, H-2, H-4), 1.92 (2H, td, J = 13.6, 3.8 Hz, H-5 Ph), 1.68 (2H, m, H-7 Ph), 1.64–1.48 (2H, m, H-Cy). 13C NMR (CDCl3, 75 MHz): δ 177.1 (COH), 171.4 (C-2), 165.2 (C-4), 149.6 (OCO2), 135.4 (C-4), 135.0 (C-5 Ph), 134.0 (C-1 Ph), 130.2 (C-3 Ph), 129.9 (C-6 Ph), 129.6 (C-2 Ph), 127.5 (C-2 Ph), 123.1 (C-3), 121.3 (C-3′), 77.3 (C-8′), 68.9 (C-6), 60.9 (C-5′), 55.8 (OMe), 33.1 (C-2), 31.5 (C-6′-C-10′), 28.3 (C-3′-C-9′), 27.3 (C-2), 20.8 and 19.1 (2 × Me-Ph); HRMS: calcld for C25H32NO7 [M + H]+, 458.2173; found, 458.2161.
HRMS: calc for C_{45}H_{52}NO_{8} [M + H]^{+}, 474.2122; found, 474.2107.

4.4. Preparation of Bioconjugates. Active ester formation was carried out with N,N-disuccinimidyl carbonate and Et_{3}N at 0 °C (Figure 1). The complete activation procedures as well as the 1H NMR spectra of the corresponding NHS esters are provided in the Supporting Information. For conjugation, a 50 mM purified hapten solution in N,N-dimethylformamide was dropwise added to a 15 mg/mL BSA or OVA solution in 100 mM phosphate buffer, pH 7.4 (50 mM carbonate—bicarbonate buffer, pH 9.6, was used to couple hapten SPo to BSA) under gentle stirring. A 5 mM purified hapten solution was slowly mixed with a 2.5 mg/mL HRP solution in 100 mM phosphate buffer, pH 7.4, for tracer enzyme conjugate preparation. BSA immunizing conjugates were prepared with a 22-fold molar excess of purified activated hapten, whereas the OVA and HRP assay conjugates were prepared with an 8-fold molar excess of hapten. The mixtures were incubated for 2 h at room temperature, and the conjugates were purified by gel filtration chromatography using 100 mM phosphate buffer, pH 7.4, as the eluent. The BSA conjugate solutions were filter-sterilized with 0.45 μm pore filter units and stored frozen at −20 °C. The OVA conjugates were stored frozen, whereas the HRP conjugates were diluted 1:1 with phosphate-buffered saline (PBS) containing 1% (w/v) BSA and stored at 4 °C.

The obtained hapten-to-protein MR was determined by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS). The sample preparation and MS analysis of bioconjugates are described in the Supporting Information.

4.5. Antibody Generation. The experimental design was approved by the Bioethics Committee of the University of Valencia. The animals were manipulated according to Spanish laws (RD1201/2005 and law 32/2007) and in compliance with the EU Directive 2010/63/EU about the protection of laboratory animals. Two groups of two female New Zealand white rabbits were subcutaneously immunized with 300 μL of an immunogen consisting of a 1:1 emulsion either BSA or OVA solution in 100 mM phosphate buffer, pH 7.4 (50 mM carbonate—bicarbonate buffer, pH 9.6, was used to couple hapten SPo to BSA) under gentle stirring. A 5 mM purified hapten solution was slowly mixed with a 2.5 mg/mL HRP solution in 100 mM phosphate buffer, pH 7.4, for tracer enzyme conjugate preparation. BSA immunizing conjugates were prepared with a 22-fold molar excess of purified activated hapten, whereas the OVA and HRP assay conjugates were prepared with an 8-fold molar excess of hapten. The mixtures were incubated for 2 h at room temperature, and the conjugates were purified by gel filtration chromatography using 100 mM phosphate buffer, pH 7.4, as the eluent. The BSA conjugate solutions were filter-sterilized with 0.45 μm pore filter units and stored frozen at −20 °C. The OVA conjugates were stored frozen, whereas the HRP conjugates were diluted 1:1 with phosphate-buffered saline (PBS) containing 1% (w/v) BSA and stored at 4 °C. The obtained hapten-to-protein MR was determined by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS). The sample preparation and MS analysis of bioconjugates are described in the Supporting Information.

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4.6. Competitive ELISA. Immunoassays were carried out in three different competitive ELISA formats. The indirect format was employed by immobilizing on the microplate wells the OVA—hapten conjugates and using an enzyme-labeled secondary antibody. The direct format was performed with antibody-coated plates and using the HRP—hapten enzyme tracers. Finally, the capture antibody direct format was evaluated with immobilized goat antirabbit immunoglobulin and the corresponding enzyme tracers in solution. Eight-point standard curves in PBS (10 mM phosphate buffer, pH 7.4, containing 140 mM NaCl) were prepared in borosilicate glass vials by serial dilution, including a blank without analyte. The ELISA absorbance values were fitted to a four-parameter logistic equation. Assay sensitivity was defined as the concentration of analyte at the inflexion point of the sigmoidal curve, typically corresponding to a 50% inhibition (IC_{50}) of the maximum signal (A_{max}). Antibody titer was defined as the antibody dilution affording an A_{max} value near to 1.0. The assay selectivity was estimated from the quotient between the IC_{50} of the reference analyte and the IC_{50} of the cross-reacting compound. For complete immunoassay procedures, see the Supporting Information.

ASSOCIATED CONTENT

Supporting Information
The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.8b01784.

General procedures, materials, and instrumentation; preparation of N-hydroxysuccinimidyl esters of hapten; MALDI-MS analysis of bioconjugates; competitive ELISA procedures; and 1H NMR spectra of the hapten's SPc and SPo and their NHS esters (PDF)

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Notes
The authors declare no competing financial interest.

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