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Rationally designed haptens for highly sensitive monoclonal antibody-based immunoanalysis of fenhexamid

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Immunochemical methods have been consolidated during the last years as complementary analytical strategies for chemical contaminant and residue determination. However, generation of suitable immunoreagents for small organic molecules demands adequate hapten design. In this study, fenhexamid was considered as a model compound and novel haptens were designed and synthesized in order to evaluate the influence of the linker tethering site on antibody binding properties and immunoassay parameters. Haptens were conceived with the spacer arm at different positions while the more antigenic aromatic moiety was kept free. The synthesis of these functionalized compounds was accomplished by total construction of the molecule through several steps. This strategy afforded very high-affinity monoclonal antibodies specific of fenhexamid, with IC₅₀ values around or below 0.1 nM. Using these novel immunoreagents, a direct competitive enzyme-linked immunosorbent assay with a remarkably low limit of detection (4 ng L⁻¹) was developed for the determination of fenhexamid residues. The selected immunoassay was investigated in terms of trueness, precision, repeatability, and robustness. The QuEChERS extraction methodology was applied to fortified samples and recoveries between 83% and 113%, with relative standard deviations below 20%, were observed. Moreover, contaminated and blind spiked samples were measured by the developed immunoassay and by ultra-performance liquid chromatography coupled to tandem mass spectrometry, showing statistically comparable results.

Introduction

1,4-Hydroxylanilides comprise a family of modern biocide compounds with varied toxicological and degradation patterns, which depend on the properties of the aromatic substituent.¹ Among this group, fenhexamid (*N*-(2,3-dichloro-4-hydroxyphenyl)-1-methylcyclohexanecarboxamide) has reached high commercial significance as fungicide due to its low persistence and high efficiency. Analysis of chemical residues and contaminants is commonly performed by chromatographic determination with spectrometry detection, commonly coupled to the QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe) extraction procedure.^{2,3} By using either gas^{4,5} or liquid^{6–8} chromatography coupled to mass spectrometry detection, limits of quantification (LOQ) in the 1–10 µg kg⁻¹ range were achieved. Although chromatographic

methods are sensitive, selective, and precise, they are time-consuming and require expensive equipment. Therefore, immunoassays have been frequently proposed as a valuable complementary method in order to improve sample throughput, thus reducing time and costs, while maintaining high analytical performance.

Antibody and bioconjugate properties determine the good performance of immunochemical methods dealing with small organic molecules, such as fenhexamid. Ultimately, immunoreagent generation with adequate binding features is strongly influenced by the structure of the immunizing chemical (hapten) that is employed in bioconjugate preparation.^{9–12} Designed haptens usually consist of a molecular scaffold mimicking the target compound, a spacer arm, and a reactive or activatable chemical group for coupling to the carrier macromolecule. Immunizing haptens should resemble as much as possible the electronic distribution and steric properties of the target substance. In general, it is recommended not to modify important immunodeterminant groups or any other moieties that are characteristic of the target compound.¹³ Introduction of the spacer arm at a distal position from those relevant moieties, as already pointed out by Nobel Prize Karl Landsteiner in 1962,¹⁴ is a well-known strategy. Linker properties, such as length, composition, and particularly the tethering site, are fundamental structural parameters which may influence the physicochemical

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Electronic Supplementary Information (ESI) available: General methods and instruments, experimental details of hapten synthesis, MALDI-TOF spectra of bioconjugates (Figure S1), checkerboard titration of antibodies (Table S1), precision of the standard curve (Table S2), matrix effects (Figure S2), determination of fenhexamid in contaminated samples by cELISA and UPLC-MS (Table S3), ¹H NMR spectra of haptens FHm, FHd and FHh and their NHS-esters. See DOI: 10.1039/x0xx00000x

Fig. 1 (A) Chemical structure of fenhexamid and the prepared haptens. (B) Electron density surface coloured by electrostatic potential of the most stable conformation of fenhexamid. The surface is coloured to reflect the electrostatic potential at every point on the surface; colour 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 colour 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. Arrows denote the spacer-arm attachment site in each of the haptens.

characteristics of the hapten, and eventually the adequate display of the molecule to the medium.

Due to incomplete knowledge about antigen-antibody interaction mechanisms, most hapten designs are based on previous scientific experience and trial-and-error approaches. However, during the last years, researchers have focused on theoretical assessment relying upon empirical results.¹⁵⁻¹⁶ Computer-assisted molecular modelling (CAMP) techniques are potent tools for rational chemical design, which may help immunochemists to discover the most suitable hapten structure for immunizing conjugate and antigen preparation. Moreover, CAMP-based structure-activity relationship analyses can contribute to better understand affinity and specificity characteristics of the produced biomolecules.¹⁷⁻¹⁸ Structurally, the fenhexamid framework is made up by an amido bridge that links an aliphatic moiety to an aromatic group (Fig. 1). The fenhexamid framework may adopt several low-energy conformations associated to the restriction of rotation around the planar amide bond and to the chair-conformational equilibrium of the cyclohexane ring. Two conformers of fenhexamid have been observed in the solid phase, by crystallization of fenhexamid in tetrahydrofuran and X-ray analysis, one with an axially oriented phenylcarbamoyl group and the other with this group in an equatorial disposition, in a ratio of 55:45.¹ The last of these conformations is similar to the minimum energy conformation calculated by Molecular Mechanics (Figure 1B). Functionalization of fenhexamid for bioconjugation can readily be accomplished by derivatization of the phenolic hydroxyl oxygen with a properly functionalized alkyl halide. With such a tethering site, the distal and less immunogenic aliphatic groups could be preferentially displayed. Moderate-affinity antibodies to fenhexamid were produced before using such strategy.¹⁹

The aim of the present study was to evaluate the structure-activity relationship of different haptens for the generation of high-affinity monoclonal antibodies to fenhexamid. Novel functionalized derivatives were conceived in which the dichlorohydroxyanilide aromatic moiety would be preferentially displayed by placing the spacer arm at the opposite site of the molecule, thus favouring π -stacking and hydrophobic interactions as well as formation of hydrogen bonds, and consequently increasing antibody affinity. Moreover, the development of a highly sensitive competitive enzyme-linked immunosorbent assay (cELISA) for the determination of fenhexamid residues in vegetables was studied. The proposed immunoassay was validated by comparison with ultra-performance liquid chromatography tandem mass spectrometry analysis (UPLC-MS/MS).

Experimental

Reagents and instrumentation

Chemical reagents used for hapten synthesis were acquired from commercial sources and used without purification. Ethyl 1,4-dioxaspiro[4.5]decane-8-carboxylate (**10**) was prepared as described by Barrios²⁰ and 4-(benzyloxy)-2,3-dichloroaniline (**4**) was prepared in three steps from 4-amino-2,3-dichlorophenol as described in the ESI.[†] Solvents were purified by distillation and, if required, they were dried according to standard methods. Primary-secondary amine (PSA) was obtained from Varian (Lake Forest, CA, USA). Fraction V bovine serum albumin (BSA) and Hybridoma Fusion and Cloning Supplement (HFCS) were purchased from Roche Applied Science (Mannheim, Germany). Horseradish peroxidase (HRP), ovalbumin (OVA), Freund's adjuvants, foetal bovine serum, *o*-phenylenediamine, and triphenyl phosphate (TPP) were purchased from Sigma/Aldrich (Madrid, Spain). P3-X63-Ag 8.653 mouse plasmacytoma cell line was from the European Collection of Cell Cultures (Wiltshire, UK). HiTrap Desalting Sephadex G-25 and HiTrap Protein G HP columns were acquired from GE Healthcare (Uppsala, Sweden) and operated under an ÄKTA Purifier workstation. Fenhexamid and other fungicide standards were purchased from Fluka/Riedel-de-Haën (Seelze, Germany) or Dr. Ehrenstorfer (Augsburg, Germany). Working standard solutions were prepared as concentrated solutions in acetonitrile and kept at -20 °C in amber glass vials. Costar™ flat-bottom high-binding polystyrene ELISA plates were from Corning (Corning, NY, USA). ELISA plates were washed with an ELx405 microplate washer from BioTek Instruments (Winooski, VT, USA). Ultraviolet-visible spectra and ELISA absorbances were read with a PowerWave HT also from BioTek Instruments. A T-25 ultra-turrax blender and a vortex mixer Ms2 from IKA (Staufen, Germany), as well as an Eppendorf 5804 centrifuge (Hamburg, Germany) were employed for the extraction of vegetables.

Molecular modelling

Calculations were performed using Molecular Mechanics (MM3) as implemented in the CAChe program [CAChe

Fig. 2 Synthesis of hapten FHm.

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.

Hapten synthesis

The haptens that were used for the preparation of immunogen and coating conjugates were synthesized according to the synthetic pathways depicted in Figs. 2 and 3. The spectroscopic characterization data of the intermediates of the synthesis are given in the ESI.[†]

Preparation of hapten FHm

Synthesis of 1-allylcyclohexanecarboxylic acid (2).²¹ A solution of acid **1** (668 mg, 5.21 mmol) in anhydrous THF (3.2 mL) was dropwise added into a 0.52 M solution of LDA in THF-hexane (24.1 mL, 13.1 mmol, 2.5 equiv; prepared from 8.2 mL of 1.6 M BuLi in hexane and 2.0 mL of diisopropylamine in 13.9 mL of THF) cooled at 0 °C under nitrogen atmosphere. The reaction mixture was stirred at this temperature for 45 min, then at room temperature (rt) for 1 h and at 60 °C for 30 min. After this time, the reaction mixture was cooled to 0 °C and then freshly distilled allyl bromide (1.108 mL, 12.0 mmol, 2.3 equiv)

was added and the mixture stirred at rt overnight. The reaction mixture was cooled to 0 °C, then quenched by the addition of a saturated aqueous solution of NH₄Cl (5 mL) and acidified with 2 M H₂SO₄ to pH 1–2. The mixture was extracted with Et₂O and the ethereal layers were extracted with a 10% (w/v) aqueous solution of Na₂CO₃. The combined aqueous layers were cooled to 0 °C, acidified to pH 3 with 6 M H₂SO₄, and extracted with Et₂O. The combined organic layers were washed with brine, dried over anhydrous MgSO₄, filtered, and concentrated under vacuum to give the acid **2** (737 mg, 84%) as an oil, which was determined to be essentially pure by ¹H NMR. This material was subsequently employed directly without further purification.

Synthesis of 1-allyl-N-(4-(benzyloxy)-2,3-dichlorophenyl)cyclohexanecarboxamide (5). A solution of acid **2** (123 mg, 0.785 mmol) in SOCl₂ (1 mL) was stirred under anhydrous conditions at reflux for 2 h. Evaporation of the excess of SOCl₂ under vacuum afforded the intermediate carboxylic acid chloride **3** (146.5 mg, 0.784 mmol), which was dissolved in anhydrous THF (2.5 mL) and cooled in a water-ice bath under nitrogen. Et₃N (328 μL, 2.35 mmol) and a solution of aniline **4** (178 mg, 0.664 mmol) in THF (2.5 mL) were then added, and the mixture stirred at rt for 5 h, then poured into water and extracted with Et₂O. The combined organic layers were washed with brine and dried over anhydrous Na₂SO₄. Chromatography of the residue left after evaporation of the solvent under reduced pressure, using hexane/EtOAc mixtures from 95:5 to 70:30 as eluent, afforded the amide **5** (183.1 mg, 66% overall yield from **2**) as an oil.

Synthesis of (E)-methyl 6-(1-((4-(benzyloxy)-2,3-dichlorophenyl)carbamoyl)cyclohexyl) hex-4-enoate (7). A solution of *p*-quinone (7 mg, 0.065 mmol), Grubbs second-generation catalyst [(1,3-dimesityl-2-imidazolylidene)(PCy₃)Cl₂RuCHPh] (18.3 mg, 0.021 mmol), methyl 4-pentenoate (73.7 mg, 0.64 mmol), and amide **5** (90 mg, 0.215 mmol) in degassed anhydrous CH₂Cl₂ (3 mL) was placed in a microwave tube under a N₂ atmosphere. The reaction mixture was then heated using a microwave oven at 60 °C for 40 min. The solvent was removed under vacuum and the obtained residue was chromatographed, using hexane/CH₂Cl₂ 1:1 as eluent, to afford, in order of elution, unreacted starting material (35.1 mg, 39%) and alkene **7** (59.7 mg, 55%; 90% based on recovered starting material). In some cases, compound **7** was obtained as a slightly coloured oil (blue), due to catalyst impurities, and a second chromatographic purification, using CH₂Cl₂ as eluent, was required to obtain a colourless product.

Synthesis of methyl 6-(1-((2,3-dichloro-4-hydroxyphenyl)carbamoyl)cyclohexyl)hexanoate (8). A mixture of compound **7** (56.8 mg, 0.112 mmol) and 10% Pd/C (8 mg) in 10:1 MeOH/CH₂Cl₂ (4.5 mL) was stirred under an atmosphere of hydrogen at 50 psi for 9 h. The reaction mixture was filtered through a short silica gel column, using MeOH as the eluent, and the residue obtained after concentration of the filtrate was chromatographed on silica gel, using CHCl₃/EtOAc 9:1 as eluent, to give the phenol **8** (36.6 mg, 78%) as a colourless oil.

Fig. 3 Synthesis of haptens FHd and FHH.

Synthesis of 6-(1-((2,3-dichloro-4-hydroxyphenyl)carbamoyl)cyclohexyl)hexanoic acid (hapten FHm). LiOH (19.2 mg, 0.457 mmol) was added to a solution of methyl ester **8** (19 mg, 0.046 mmol) in a mixture of THF (360 μ L) and H₂O (160 μ L). The resulting reaction mixture was stirred at rt for 3 h, diluted with water, and acidified with an aqueous solution of KHSO₄. The mixture was extracted with Et₂O and the combined organic layers were washed with brine, dried over anhydrous MgSO₄, and concentrated under vacuum to give hapten FHm (17.9 mg, 97.3%) as a white solid. Mp 133–137 °C (from cold MeOH); IR ν_{max} (cm⁻¹) (KBr) 3500–2500, 3412, 3320, 2931, 1701, 1654, 1518, 1284; ¹H NMR (300 MHz, CD₃OD) δ (ppm) 7.17 (1H, d, *J* = 8.8 Hz, H-6 Ph), 6.89 (1H, d *J* = 8.8 Hz, H-5 Ph), 2.28 (2H, t, *J* = 7.3 Hz, H-2), 2.16 (2H, H-2/H-6 Cy), 1.7–1.5 (8H, m), 1.45–1.3 (8H, m); ¹³C NMR (75 MHz, THF-*d*₈) δ (ppm) 174.7 (CO₂H), 174.6 (CON), 152.6 (C4-Ph), 129.9 (C1-Ph), 127.1 (C2-Ph), 125.2 (C6-Ph), 120.2 (C3-Ph), 115.3 (C5-Ph), 48.1 (C1-Cy), 35.5 (C2/C6-Cy), 34.4 (C6), 34.3 (C2), 30.0 (C4), 27.3 (C4-Cy), 25.9 (C3), 24.9 (C5), 24.2 (C3/C5-Cy); HRMS (TOF MS ES⁻) calcd for C₁₉H₂₄Cl₂NO₄ [M-H]⁻ 400.1082, found 400.1082. UV (100 mM sodium phosphate buffer, pH 7.4), ϵ (280 nm) = 1.82 mM⁻¹ cm⁻¹.

Preparation of haptens FHd and FHh

Synthesis of ethyl 8-methyl-1,4-dioxaspiro[4.5]decane-8-carboxylate (11). A solution of ester **10** (1.182 g, 5.51 mmol) in anhydrous THF (8 mL) was added dropwise over 1 h into a solution of LDA [prepared from 4.6 mL of a 1.56 M solution of BuLi in hexanes (7.17 mmol, 1.3 equiv) and 1 mL of diisopropylamine (0.726 mg, 7.17 mmol, 1.3 equiv)] in THF (8 mL) at –78 °C under argon atmosphere. The reaction mixture was stirred at the same temperature for 1.5 h, allowed to warm slowly to –15 °C, and then stirred for a further 30 min. The reaction mixture was cooled again to –78 °C, then treated with CH₃I (1.56 g, 0.69 mL, 11.02 mmol, 2 equiv), stirred at this temperature for 1 h, and then allowed to be gradually warmed to rt and stirred overnight, after which, it was poured into water and extracted with Et₂O. The combined organic layers were washed with brine, dried over anhydrous MgSO₄, filtered, and concentrated under vacuum to yield an oil that was purified by column chromatography, using a mixture of hexane/EtOAc (from 100:0 to 80:20) as eluent, to give compound **11** (1.082 g, 86%) as a colourless oil.^{22–24}

Synthesis of 8-methyl-1,4-dioxaspiro[4.5]decane-8-carboxylic acid (12). A solution of ethyl ester **11** (364.6 mg, 1.597 mmol) in a mixture of EtOH (7.3 mL) and a 5 M aqueous solution of NaOH (0.8 mL, 4.0 mmol, 2.5 equiv) was stirred at 60 °C for 3.5 h. After cooling to rt, the reaction mixture was diluted with water, extracted with Et₂O, and the aqueous layer cooled in an ice-water bath and carefully acidified with an aqueous solution of KHSO₄ to pH 4–5. The mixture was extracted with Et₂O and the combined organic layers were washed with water and brine, dried over anhydrous Na₂SO₄, and concentrated under reduced pressure to afford the acid **12** (262.5 mg, 82%) as a foam that was used in the next step without further purification.

Synthesis of N-(4-(benzyloxy)-2,3-dichlorophenyl)-8-methyl-1,4-dioxaspiro[4.5]decane-8-carboxamide (13). A 2 M solution of oxalyl chloride in CH₂Cl₂ (1.13 mL, 2.26 mmol, 3 equiv) was added to a stirred solution of acid **12** (150 mg, 0.749 mmol) in anhydrous benzene (2 mL) under nitrogen. The mixture was stirred at rt for 2 h, then an additional amount of the 2 M solution of oxalyl chloride in CH₂Cl₂ (1.13 mL) was added and the stirring was continued for 3 h. The reaction mixture was diluted with benzene and washed with a saturated aqueous solution of NaHCO₃ and brine, dried over anhydrous Na₂SO₄, and concentrated under vacuum to give the corresponding acid chloride (142 mg). This acid chloride was dissolved in anhydrous THF (3 mL) under nitrogen and the solution cooled to 0 °C. Et₃N (272 μ L, 1.95 mmol, 3 equiv) was added dropwise, followed by the dropwise addition of a solution of aniline **4** (549 mg, 2.05 mmol, 1.05 equiv) in THF (1 mL). The reaction mixture was stirred at 0 °C for 1 h and then at rt overnight. Then, it was diluted with benzene, washed with water and brine, and dried over anhydrous MgSO₄. Chromatography of the residue that was left after evaporation of the solvent under vacuum, using a mixture of hexane/EtOAc (from 100:0 to 80:20) as eluent, afforded amide **13** (286.7 mg, 85% for the two steps) as an oil.

Synthesis of N-(4-(benzyloxy)-2,3-dichlorophenyl)-1-methyl-4-oxocyclohexanecarboxamide (14). 1 M hydrochloric acid (2.5 mL) was added to a solution of ketal **13** (267.1 mg, 0.593 mmol) in THF (4 mL) and the mixture stirred at 60 °C for 2 h. The reaction mixture was cooled down to rt and diluted with Et₂O, washed with brine, dried over anhydrous MgSO₄, and concentrated at reduced pressure to give a residue that was purified by chromatography, using a hexane/EtOAc from 80:20 to 60:40 as eluent, to give ketone **14** (197.6 mg, 82%) as a white solid.

Synthesis of 5-(4-((4-(benzyloxy)-2,3-dichlorophenyl)carbamoyl)-4-methylcyclohexylidene)pentanoic acid (16). A 0.5 M solution of potassium *bis*(trimethylsilyl)amide in toluene (4.96 mL, 2.48 mmol, 4 equiv) was dropwise added to a solution of (4-carboxybutyl)triphenylphosphonium bromide (550 mg, 1.24 mmol) in anhydrous THF (5 mL) at 0 °C under nitrogen. The resulting orange mixture was stirred for 15 min at this temperature before a solution of ketone **14** (251.8 mg, 0.620 mmol) in hexamethylphosphorotriamide (2.5 mL) was added dropwise. The mixture was stirred for 1.5 h and then poured into water, cooled to 0 °C, acidified with 1 M hydrochloric acid to pH 2, and extracted with EtOAc. The combined organic layers were washed with brine, dried over anhydrous Na₂SO₄, and concentrated under vacuum. Chromatographic purification, using hexane/EtOAc 7:3 as eluent, afforded **16** (213 mg, 70%) as a semisolid.

Synthesis of 5-(4-((2,3-dichloro-4-hydroxyphenyl)carbamoyl)-4-methylcyclohexylidene)pentanoic acid (hapten FHd). A 1 M solution of BCl₃ in CH₂Cl₂ (420 μ L, 0.420 mmol, 2.1 equiv) was dropwise added during 5 min to a solution of benzyl ether **16** (100 mg, 0.200 mmol) and pentamethylbenzene (90.7 mg, 0.600 mmol, 3 equiv) in anhydrous CH₂Cl₂ (3.2 mL) at –78 °C under nitrogen atmosphere. The reaction mixture was stirred

at the same temperature for 10 min and then quenched by the addition of a 4:1 mixture of THF and an aqueous solution of NH_4Cl (10 mL). The mixture was extracted with ethyl ether and the combined ethereal layers were washed with brine and dried over anhydrous MgSO_4 . Chromatographic purification of the residue that was left after evaporation of the solvent, using $\text{CHCl}_3/\text{MeOH}$ 95:5 as eluent, afforded hapten FHd (78 mg, 95%) as a colourless thick oil. IR ν_{max} (cm^{-1}) (NaCl) 3500–2500, 2978, 2847, 1706, 1656, 1498, 1411, 1274, 1181, 946; ^1H NMR (300 MHz, CDCl_3) δ (ppm) 7.93 (1H, d, J = 9 Hz, H-6 Ph), 7.76 (1H, br s, NH), 6.90 (1H, d, J = 9 Hz, H-5 Ph), 5.12 (1H, t, J = 7.5, H-5), 2.34 (3H, m, 1H Cy, overlapped with t, J = 7.5 Hz, H-2), 2.24–2.06 (5H, m, 5H Cy), 2.05 (2H, dt, J = 7.5, 7.5 Hz, H-4), 1.68 (2H, quint, J = 7.3 Hz, H-3), 1.52 (2H, m, 2H Cy), 1.32 (3H, s, CH_3); ^{13}C NMR (75 MHz, CDCl_3) δ (ppm) 179.5 (COOH), 175.9 (CON), 149.5 (C4-Ph), 138.2 (C1-Cy), 128.4 (C1-Ph), 123.6 (C2-Ph), 122.3 (C6-Ph), 121.3 (C-5), 119.1 (C3-Ph), 114.6 (C5-Ph), 44.3 (C4-Cy), 37.0 (C3-Cy), 36.2 (C5-Cy), 33.2 (C2), 32.9 (C2-Cy), 26.4 (C-4), 26.0 (CH_3), 24.8 (C3), 24.7 (C6-Cy); HRMS (TOF MS ES+) calcd for $\text{C}_{19}\text{H}_{23}\text{Cl}_2\text{NO}_4$ $[\text{M}+\text{H}]^+$ 400.1077, found 400.1074. UV (100 mM sodium phosphate buffer, pH 7.4), ϵ (280 nm) = $3.62 \text{ mM}^{-1} \text{ cm}^{-1}$.

Synthesis of 5-(4-((2,3-dichloro-4-hydroxyphenyl)carbamoyl)-4-methylcyclohexyl)pentanoic acid (hapten FHh). A mixture of hapten FHd (21 mg, 0.052 mmol) and 5% Rhodium on alumina (11 mg) in dry EtOAc (1.5 mL) was stirred under hydrogen (55 psi) at rt for 6 h, after which the black suspension was filtered through a short pad of silica gel, using EtOAc as eluent, to afford the hapten FHh (20 mg, 95%) as an approximately 60:40 mixture of epimers at the newly generated tertiary carbon atom. IR ν_{max} (cm^{-1}) (KBr) 3500–2500, 3415, 3322, 2929, 2853, 1700, 1651, 1514, 1411, 1285, 941, 815; ^1H NMR (300 MHz, CDCl_3) (only signals of the major epimer are given) δ (ppm) 7.97 (1H, d, J = 9.1 Hz, H-6 Ph), 7.75 (1H, br s, NH), 6.91 (1H, d, J = 9.1 Hz, H-5 Ph), 2.33 (2H, t, J = 7.4 Hz, H-2), 2.21 (2H, m, H-3/H-5 Cy), 1.80–1.53 (6H, m, H-3, H-5 and 2H Cy), 1.38–1.07 (7H, m, C-4, 5H Cy), 1.25 (3H, s, CH_3); ^{13}C NMR (75 MHz, CDCl_3) (only signals of the major epimer are given) δ (ppm) 179.4 (COOH), 175.6 (CON), 149.4 (C4-Ph), 128.6 (C1-Ph), 123.2 (C2-Ph), 122.1 (C6-Ph), 119.0 (C3-Ph), 114.6 (C5-Ph), 44.5 (C4-Cy), 36.8 (C1-Cy), 36.2 (C3/C5-Cy), 33.9 (C2), 33.7 (C2/C6-Cy), 30.0 (C5), 29.4 (CH_3), 26.2 (C4), 24.8 (C3). HRMS (TOF MS ES-) calcd for $\text{C}_{19}\text{H}_{24}\text{Cl}_2\text{NO}_4$ $[\text{M}-\text{H}]^-$ 400.1088, found 400.1091. UV (100 mM sodium phosphate buffer, pH 7.4), ϵ (280 nm) = $1.66 \text{ mM}^{-1} \text{ cm}^{-1}$.

Hapten activation and bioconjugation

The three synthetic haptens were activated by formation of the corresponding *N*-hydroxysuccinimidyl ester using *N,N'*-disuccinimidyl carbonate and Et_3N in dry acetonitrile as previously described.²⁵ Confirmation of structure and purity of these active esters was obtained by ^1H NMR spectroscopic analysis. A more detailed description of the activation reaction and copies of the original spectra are included in the ESI.[†]

Conjugation of haptens to BSA, OVA, and HRP was carried out as previously described.²⁵ BSA conjugates were employed for immunization, OVA conjugates for hybridoma screening

assays, and HRP tracers for immunoassay development. Matrix-Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF) was performed with a 5800 MALDI-TOF/TOF (ABSciex, Framingham, MA, USA) in positive linear mode (1500 shots every position) in a mass range of 15000–130000 m/z . Every sample was calibrated by close external calibration with a BSA, OVA, or HRP spectrum. The corresponding MALDI spectra are supplied in the ESI.[†]

Monoclonal antibody generation

Animal manipulation was carried out 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 A1248961469416). Immunogen preparation and mouse immunization were performed according to regular procedures as previously published.²⁶ Hybridomas were generated, selected, and cloned as described elsewhere.²⁷ For details see the ESI.[†] Immunoglobulins were purified from late stationary phase culture supernatants by ammonium sulphate precipitation and protein G affinity chromatography.

Competitive ELISA

Ninety-six-well ELISA plates were coated with 100 μL per well of a particular antibody solution in 50 mM carbonate–bicarbonate buffer, pH 9.6, and plates were incubated overnight at rt. Coated plates were washed four times with 150 mM NaCl containing 0.05% (v/v) Tween 20 and received, sequentially, 50 μL per well of analyte standard solution in PBS (10 mM sodium phosphate buffer, pH 7.4, with 140 mM NaCl) plus 50 μL per well of enzyme tracer solution in PBST (PBS containing 0.05% (v/v) Tween 20). The immunological reaction took place during 1 h at rt, and then plates were washed as described before. Finally, retained peroxidase activity was determined by adding 100 μL per well of freshly prepared 2 g L^{-1} *o*-phenylenediamine solution containing 0.012% (v/v) H_2O_2 in 25 mM citrate and 62 mM sodium phosphate buffer, pH 5.4. The enzymatic reaction was stopped after 10 min at rt with 100 μL per well of 1 M sulfuric acid. The absorbance was immediately read at 492 nm with a reference wavelength at 650 nm.

Eight-point standard curves were prepared from a 2 $\mu\text{g L}^{-1}$ fenhexamid solution by four-fold serial dilution in PBS, including a blank with no analyte. Experimental values were fitted to a four-parameter logistic equation using the SigmaPlot software package from SPSS Inc. (Chicago, IL, USA). Assay sensitivity was defined as the concentration of analyte at the inflection point of the fitted sigmoidal curve, typically corresponding to a 50% reduction (IC_{50}) of the maximum absorbance (A_{max}) if the background signal approaches to zero. Deming regression from the SigmaPlot v.12.0 software was applied for method validation.

Solvent and buffer studies

Solvent effects over the proposed immunoassay were evaluated using fenhexamid standard curves that had been prepared in the presence of ethanol or acetonitrile at different

concentrations (from 0.5 to 10% (v/v) in Milli-Q water). Then, the corresponding immunoreagent solution was prepared in 20 mM sodium phosphate buffer, pH 7.4, containing 280 mM NaCl and 0.05% (v/v) Tween 20 in order to complete the immunoassay. The influence of buffer pH over the inhibition curve of the cELISA was evaluated using buffers with pH values between 5.5 and 9.5, which were prepared as reported elsewhere.²⁵

Sample treatment

Tomato and cucumber plants were grown in a greenhouse and treated with Teldor – a wettable powder containing 50% (w/w) fenhexamid – following the manufacturer's instructions (<http://www.cropscience.bayer.com/>). After the application of the commercial product, fruits were collected at different times (from 1 to 14 days) in order to obtain samples with different contents of fungicide, and they were short-term stored at 4 °C. Then, samples were chopped, homogenized using a blender, and stored in polypropylene tubes at –20 °C until analysis.

QuEChERS method was employed for the extraction and purification of fenhexamid residues from vegetables.² Five grams of chopped vegetable plus 50 µL of internal standard (TPP, 50 mg L⁻¹) were introduced in a 50 mL polypropylene centrifuge tube containing 0.5 g of sodium acetate and 2 g of anhydrous magnesium sulphate. Sample was then extracted with 5 mL of 1% (v/v) acetic acid in acetonitrile by vortexing 1 min, and centrifuged for 5 min at 2200×g. Then, 1 mL of extract was introduced into a 2 mL polypropylene centrifuge tube containing 50 mg of PSA and 150 mg of anhydrous magnesium sulphate, vortexed during 1 min, and centrifuged for 5 min at 2200×g. Finally, the purified extract was filtered through a 0.22 µm Teflon filter and analysed by the optimized cELISA and also by a reference chromatographic procedure.

Chromatographic analysis

Ultra-performance liquid chromatography coupled to a triple quadrupole mass spectrometer was selected as reference methodology in order to obtain maximum sensitivity and selectivity for the analysis of fenhexamid residues. Chromatography was performed with a Waters Acquity UPLC system (Milford, MA, USA) equipped with a binary solvent delivery system, an autosampler, and a BEH C18 (1.7 µm, 2.1 × 50 mm) column. The injection volume was set at 5 µL and the mobile phase consisted of 0.5% (v/v) formic acid in water (A) and acetonitrile (B). The gradient started at 30% of B, it was linearly increased to 85% in 3.4 min, and a second ramp to 98% of B in 1.7 min at a flow rate of 350 µL min⁻¹. The obtained retention times using aforementioned conditions were 3.18 and 3.85 min for fenhexamid and TPP, respectively. Tandem mass acquisitions were performed in a Waters Acquity triple quadrupole mass spectrometry detector, equipped with a Z-spray electrospray ionization source, with 3.5 kV as capillary voltage, 120 °C as source temperature, and 300 °C as desolvation temperature. The employed parameters were: ESI+, parent ion 302 m/z, daughter ions 97 and 55 m/z, 20 eV collision energy, and 35 V cone energy for fenhexamid; and

ESI+, parent ion 328 m/z, daughter ions 77 and 152 m/z, 30 eV collision energy, and 25 V cone energy for TPP.

Results and discussion

Hapten design

The fenhexamid molecule is constituted by a dichlorophenolic moiety – electronically rich, polar, and relatively hydrophilic – and a 1-methylcyclohexane group – apolar and lipophilic – attached via an amide bridge. In previous studies,¹⁹ we have obtained antibodies to fenhexamid using, as immunogen, a conjugate between BSA and hapten FHo (Fig. 1), which held the hydrocarbon spacer arm at the phenolic oxygen atom via a C–O–C ether linkage. The use of this immunogenic hapten resulted in the generation of specific antibodies against fenhexamid, in which, presumably, the distal apolar part of the fenhexamid skeleton, *i.e.*, the 1-methylcyclohexane moiety, must represent a key recognition element, and Van der Waals forces and hydrophobic interactions at the binding site make, most likely, a very substantial contribution to the binding energy.

For the present study, new haptens were designed in order to evaluate the immunizing activity and the properties of the generated antibodies when alternative derivatization positions were used (Fig. 1). In hapten FHm, the carboxylate-containing side chain that constituted the spacer arm was placed at a central position of the fenhexamid molecule, by the formal replacement of a C–H methyl bond by a C–C bond, whereas in hapten FHh, the chain was located at the farthest possible end of the polar part of the molecule, the C-4 position of the cyclohexane ring, also through a C–C linkage. No major modification of the electronic and conformational properties of the fenhexamid framework occurred in these haptens, given the nature of the linkage of the side chain and the tethering sites. In both cases, display of the main antigenic determinant of the fenhexamid molecule – the dichlorophenolic moiety – was theoretically maximized. This antigen presentation mode was complementary to that of hapten FHo. In principle, the location of the linker in the newly designed haptens should favour the interaction with the distal aromatic and most substituted part of the fenhexamid framework, and could allow the generation of antibody binding sites in which the bimolecular interaction is strongly stabilized by additional forces, such as hydrogen bonding and electrostatic or π -stacking interactions, thus increasing the antibody affinity. Additionally to the above mentioned haptens, hapten FHd – which was a precursor of the synthesis of hapten FHh – was also included in this study. In this hapten, the linker was joined at the C-4 position of the cyclohexane ring through a rigid C=C double bond which limited the conformational flexibility of both the linker chain and the cyclohexane ring (Fig. 1).

Hapten synthesis

The common fenhexamid framework of the designed haptens was constructed following a single strategy, *i.e.*, the initial preparation of a conveniently substituted cyclohexane

carboxylic acid which was coupled with the amino-dichlorophenolic moiety through an amidation reaction. The synthesis of hapten FHm was initiated with the alkylation reaction of the lithium enolate derived from carboxylic acid **1** with allyl bromide to give allyl derivative **2** (Fig. 2). Condensation of this – through the corresponding acyl chloride derivative **3** – with the aniline **4** led to amide **5**, which possessed the complete fenhexamid framework and an allyl double bond that was used in the subsequent steps to complete the elaboration of the hydrocarbon chain linker. Thus, microwave assisted cross-metathesis reaction of the double bond of **5** with methyl 4-pentenoate (**6**), catalysed by a ruthenium–carbene complex (Grubbs 2nd generation catalyst), followed by catalytic hydrogenation of the double bond and concomitant hydrogenolysis of the benzyl ether moiety of **7** afforded the methyl ester **8**, whose hydrolysis under standard basic conditions completed the synthesis of hapten FHm. The synthesis of this hapten from readily available carboxylic acid **1** involved six synthetic steps with an overall yield of about 30%.

The synthesis of haptens FHd and FHH started from ethyl keto-carboxylate **9**, which, after protection of the ketone carbonyl group as the corresponding ethylene ketal, was methylated through the corresponding lithium enolate to give **11** (Fig. 3). Completion of the fenhexamid framework was readily achieved through basic hydrolysis of the ester moiety followed by transformation of the obtained acid **12** to the corresponding acyl chloride and reaction of this with the aniline **4**. Acid catalysed hydrolysis of the ethylene ketal group of **13**, followed by introduction of the hydrocarbon spacer arm by means of a Wittig olefination reaction, afforded compound **16**, which was selectively debenzylated under very mild conditions to give hapten FHd. Catalytic hydrogenation of this, using rhodium over aluminum oxide as catalyst to avoid hydrogenolysis of the C–Cl bond, completed the synthesis of hapten FHH, which was obtained as a 60:40 mixture of epimers at the newly generated stereogenic centre. Both epimers could not be separated by the usual chromatographic purification techniques and the mixture was used as such in the preparation of the corresponding protein conjugates. The synthesis of haptens FHd and FHH from starting keto-carboxylic ester **9** required seven and eight synthetic steps, with a global yield of 31% and 30%, respectively.

Bioconjugate evaluation

The purified active esters of the synthetic haptens were used for covalent coupling to the carrier proteins. Final hapten-to-protein molar ratios (MR) were determined by MALDI-TOF/MS (Fig. S1), and also by differential absorbance at 280 nm considering that the extinction coefficients of the hapten and the protein remained unaltered after conjugation. Determinations by both methods were generally comparable (Table 1), except for the HRP conjugates whose MR were inconsistent by UV analysis since only 3 lysine residues of this enzyme are available for conjugation.²⁸ In general, the efficiency of the conjugation reaction to BSA and OVA was higher for the conjugates prepared in the present study – haptens FHm, FHd, and FHH – by using the purified active

ester; as compared to the corresponding conjugates of hapten FHo from a previous study¹⁹ in which the one-pot reaction strategy was followed for hapten coupling. Consequently, lower amounts of hapten were required when the purified active ester was used. Moreover, the final MR could be finely tuned as exemplified by the low hapten densities of the novel OVA conjugates.

Table 1 Hapten-to-protein molar ratio of the prepared conjugates

Conjugate	MR ^a	MALDI	UV
BSA-FHm	30	13.4	15
BSA-FHd	30	24.0	12
BSA-FHH	30	19.8	23
BSA-FHo	74	20.0	20
OVA-FHm	3	0.9	3
OVA-FHd	3	2.2	4
OVA-FHH	3	1.6	2
OVA-FHo	13	7.0	10
HRP-FHm	10	0.7	6
HRP-FHd	10	1.4	3
HRP-FHH	10	1.2	4
HRP-FHo	18	2.0	2

^a Hapten-to-protein molar ratio in the coupling mixture.

Immunizing activity of haptens

Mice were immunized with the BSA conjugate of haptens FHm, FHd, and FHH, and novel monoclonal antibodies were raised. All of these immunoglobulins were of the IgG₁ isotype and contained a κ light chain. In a previous study, monoclonal antibodies had been generated from hapten FHo.¹⁹ The complete collection of monoclonal antibodies to fenhexamid – including those produced earlier – was evaluated by checkerboard direct cELISA using the corresponding homologous tracer (Table 2). We found that monoclonal antibodies with very low IC₅₀ values (around or below 0.1 nM) could be obtained from conjugates of the three novel haptens. The position of the linker probably determined that the affinity of the novel antibodies was higher than that of the antibodies

Table 2 Monoclonal antibody characterization (n=3)^a

mAb	[T] ^b	A _{max}	IC ₅₀ ^c
FHh#15	30	1.19	0.04
FHh#122	100	0.92	0.08
FHh#130	30	0.85	0.08
FHd#19	3	0.82	0.60
FHd#132	30	1.27	0.08
FHd#139	10	1.56	0.12
FHm#110	30	1.63	0.14
FHm#113	10	0.86	0.05
FHm#114	100	1.02	0.02
FHo#22	30	1.92	0.93
FHo#26	10	1.29	0.66
FHo#27	10	1.55	0.69

^a Antibody coating concentration was 1000 ng mL⁻¹. ^b Tracer concentration in ng mL⁻¹. ^c Values are in nM units.

Fig. 4 Standard inhibition curve for the proposed cELISA. Plates were coated with a 1000 ng mL⁻¹ FHM#114 antibody solution and tracer HRP-FHh was employed at 30 ng mL⁻¹. Results are the mean of five independent experiments. Curve parameters were: $A_{\max} = 98 \pm 3\%$, slope = -1.32 ± 0.09 , $IC_{50} = 0.022 \pm 0.002 \mu\text{g L}^{-1}$, lower asymptote = $0 \pm 2\%$, and $R^2 = 0.998$.

generated previously from hapten FHo. Interestingly, the outstanding affinity values of antibodies that were raised with haptens FHh and FHD was independent of the double bond at the linker tethering site. More surprisingly, extraordinary affinities were also displayed by antibodies generated from hapten FHM, with a central tethering site closer to the dichlorophenolic moiety. All of the monoclonal antibodies were specific of fenhexamid. Twenty-two fungicides (pyraclostrobin, kresoxim-methyl, trifloxystrobin, azoxystrobin, dimoxystrobin, fluoxastrobin, metominostrobin, picoxystrobin, boscalid, captan, cyprodinil, mepanipyrim, pyrimethanil, procymidone, tolylfluanid, cyazofamid, tebuconazole, fenamidone, fludioxonil, vinclozolin, imidacloprid, and benzanilide) were evaluated as competitors. Calibration curves were prepared up to 10 μM for each compound in PBS and no inhibition was observed.

Immunoassay selection and characterization

The antibody–tracer combination providing the highest sensitivity with moderate immunoreagent consumption for a satisfactory signal at zero dose of analyte, was selected by checkerboard cELISA using homologous and heterologous enzyme tracers (Table S1). On these bases, antibody FHM#114 and the heterologous tracer HRP-FHh were selected for further immunoassay development. The standard inhibition

curve of the proposed cELISA is shown in Fig. 4. The $A_{\max}$ value of the sigmoidal curve was 1.3 absorbance units, the slope was -1.32 , the IC_{50} value was 20 ng L^{-1} , and the background signal was close to zero. Moreover, the limit of detection (LOD), estimated as the fenhexamid concentration that provided a 10% reduction of the $A_{\max}$, was 4 ng L^{-1} . Precision of the inhibition curve was determined as the relative standard deviation of $A_{\max}$, slope, and IC_{50} values in repetitive experiments (Table S2). Both for intra-day and for inter-day determinations, precision results were lower than 10%.

Influence of solvents and buffer pH

The influence of acetonitrile and ethanol over the $A_{\max}$ and IC_{50} values of the standard curve was assessed in order to study the tolerance to solvents of the studied cELISA. Acetonitrile was evaluated because it is employed in the QuEChERS methodology, whereas ethanol is present in alcoholic drinks and in fermented food samples. It was found that both solvents were quite well tolerated (Fig. 5). Acceptable variations of $A_{\max}$ values (between $\pm 20\%$) were observed with 10% and 5% acetonitrile and ethanol, respectively. However, tolerable variations of the IC_{50} value only occurred at solvent concentrations below 2%. Besides, the effect of buffer pH over the main inhibition curve parameters of the proposed cELISA was investigated. A strong dependence of $A_{\max}$ and IC_{50} values with the buffer pH was evidenced, which was consistent with the acid dissociation constant of fenhexamid ($pK_a = 7.3$).

Determination of fenhexamid residues in vegetables

Tomato and cucumber were selected as model samples in order to study the interferences of vegetable matrices over the inhibition curve parameters of the developed cELISA. Matrix effects were evaluated using fenhexamid-free samples (measured by UPLC-MS/MS), which were extracted by the QuEChERS procedure and then diluted with Milli-Q water (1/5, 1/15, 1/50, 1/150, and 1/500). A fenhexamid standard curve was prepared with every diluted sample extract, and assayed in coated microplates and with the corresponding tracer solution in 20 mM sodium phosphate buffer, pH 7.4, containing 280 mM NaCl and 0.05% (v/v) Tween 20. Similar matrix effects were observed with both vegetables (Fig. S2).

Recovery studies were performed using the same analyte-free samples. Vegetables were processed as described in the

Fig. 5 Influence of acetonitrile, ethanol, and buffer pH changes over the $A_{\max}$ and IC_{50} values of the studied cELISA.

Table 3 Recoveries from spiked tomato and cucumber samples

[FH] ^a ($\mu\text{g kg}^{-1}$)	Dilution	Recovery (% $\pm$ s, n=3)	
		Tomato	Cucumber
0.5	1/50	95 $\pm$ 5	95 $\pm$ 6
1	1/50	89 $\pm$ 20	100 $\pm$ 6
5	1/50	83 $\pm$ 7	105 $\pm$ 13
10	1/500	98 $\pm$ 8	87 $\pm$ 8
50	1/500	98 $\pm$ 14	92 $\pm$ 6
100	1/500	90 $\pm$ 11	96 $\pm$ 18
500	1/5000	93 $\pm$ 12	88 $\pm$ 8
1000	1/5000	100 $\pm$ 8	113 $\pm$ 16

^a Fenhexamid concentration.

Experimental section, spiked with fenhexamid at different concentrations, and homogenized by vortex mixing. Fortified samples were extracted by QuEChERS and measured by the developed cELISA. Excellent recoveries with RSD lower than 20% were obtained in both sorts of foodstuff (Table 3). From these results, the LOQ – defined as the minimal concentration that is possible to measure with adequate accuracy and precision – could be established at $0.5 \mu\text{g kg}^{-1}$.

The developed immunoassay was validated by comparison with a reference chromatographic method using in-field treated and blind spiked samples. Tomato and cucumber crops were sprayed with a commercial fenhexamid preparation following good agricultural practices. Vegetables were collected at different moments and homogenized, and the QuEChERS-based extracts were analysed by the developed cELISA and by UPLC–MS/MS. As listed in Table S3, the fenhexamid concentration that was found for in-field treated samples ranged from 28 to $1000 \mu\text{g kg}^{-1}$ – equal or lower than the EU maximum residue limit ($1000 \mu\text{g kg}^{-1}$; http://ec.europa.eu/sanco_pesticides/). In addition, spiked blind samples were prepared in order to obtain a higher number of positive samples that could be useful for immunoassay validation with a reference method. A total of 8 samples of each foodstuff were spiked by an external operator and analysed (Table S3). For accurate method comparison, Deming regression was employed. Fig. 6 shows the regression

Fig. 6 Deming regression between the developed cELISA and UPLC–MS/MS with 95% confidence intervals.

curve for the developed immunoassay against UPLC–MS/MS with 95% confidence interval (CI) bands. The slope was 1.03 (CI from 0.93 to 1.14) and the intercept was -1.5 (CI from -6.9 to 3.9). Therefore, since the slope and intercept values were statistically equal to 1 and 0, respectively, the immunochemical results and those obtained by UPLC–MS/MS were comparable.

Conclusions

Accurate hapten design can help to improve antibody binding properties. Particularly, the linker tethering site has been demonstrated as a determinant factor for high-affinity and specific antibody generation. Three new functionalized haptens of fenhexamid have been prepared by total synthesis and protein conjugates were produced. Purification of the active ester for conjugation was shown to increase the coupling efficiency, thus reducing the amount of required hapten. A collection of monoclonal antibodies from these novel haptens was obtained, most of them displaying outstandingly low IC_{50} values (below 0.1 nM). Based on one of these monoclonals, a direct immunoassay has been characterized for the determination of fenhexamid residues in vegetables. QuEChERS-based extracts were directly diluted in water and analysed by the developed cELISA, affording excellent results from fortified, in-field treated, and blind spiked tomato and cucumber samples, with a LOQ of $0.5 \mu\text{g kg}^{-1}$. Moreover, this immunoassay showed excellent trueness, precision, and robustness, and afforded results that were statistically comparable to those of a chromatographic reference procedure. The proposed immunoassay is a straightforward and rapid method that combines high sensitivity, selectivity, and sample throughput for the analysis of fenhexamid in foodstuffs.

Conflicts of interest

There are no conflicts to declare.

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The importance of an optimum hapten design for developing highly sensitive immunoassays has been demonstrated.

Supplementary Information

Rationally designed haptens for highly sensitive monoclonal antibody-based immunosensing of fenhexamid

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

	<u>Page</u>
General information	2
Preparation of 4-(benzyloxy)-2,3-dichloroaniline (4)	2
Characterization data of intermediates of the synthesis of hapten FH <i>m</i>	3
Characterization data of intermediates of the synthesis of haptens FH <i>d</i> and FH <i>h</i>	5
Hapten activation and conjugation	7
MALDI-TOF spectra of bioconjugates (Figure S1)	8
Monoclonal antibody generation	8
Checkerboard titration (Table S1)	9
Precision of the standard curve (Table S2)	10
Matrix effects (Figure S2)	10
Determination of fenhexamid in vegetables by cELISA and UPLC-MS (Table S3)	10
¹ H NMR spectra of haptens FH <i>m</i> , FH <i>d</i> and FH <i>h</i> and their NHS-esters	11

General information

Reactions involving air-sensitive compounds were conducted in oven-dried glassware under a nitrogen atmosphere. 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). Reactions were monitored with the aid of thin-layer chromatography (TLC) using 0.25 mm pre-coated silica gel plates. Visualization was carried out with UV light and aqueous ceric ammonium molybdate solution. Melting points were determined using a Kofler hot-stage apparatus or a Büchi melting point apparatus and are uncorrected. NMR spectra were recorded at room temperature on a Bruker AC-300 spectrometer (300.13 MHz for ^1H and 75.47 MHz for ^{13}C). The spectra were referenced to residual solvent protons in ^1H NMR spectra (7.26 ppm, 3.58 for THF- d_8 and 3.31 for CD_3OD) and to solvent carbons in ^{13}C NMR spectra (77.00 ppm and 67.57 for THF- d_8). Carbon substitution degrees were established by DEPT pulse sequences. The abbreviation used for NMR data are as follows: s = singlet, d = doublet, ddd = double double doublet, t = triplet, q = quadruplet, dt = double triplet, quint = quintuplet, br = broad, m = multiplet; Cy = cyclohexyl ring, Ph = phenyl ring, Bn = aromatic benzyl ring. Infrared (IR) spectra were measured using a Nicolet Avatar 320 FT-IR spectrometer. High resolution mass spectra (HRMS) were recorded by the electrospray (ES) ionization mode using a Q-TOF premier mass spectrometer with an electrospray source (Waters, Manchester, UK).

Preparation of 4-(benzyloxy)-2,3-dichloroaniline (4)

a) Acetic anhydride (589 μL , 6.24 mmol, 1.1 equiv) was dropwise added to a well stirred solution of 4-amino-2,3-dichlorophenol (1.00 g, 5.67 mmol) in THF (10 mL) and H_2O (5 mL) at room temperature. The reaction mixture was stirred at this temperature for 1.30 h and then poured into water and extracted with Et_2O . The combined organic layers were washed with an aqueous saturated solution of NaHCO_3 and brine and dried over anhydrous Na_2SO_4 . Evaporation of the solvent at reduced pressure led to crude acetamide ii (1.199 mg, 96%).

b) Benzyl bromide (1.3 mL, 10.9 mmol, 2 equiv) was added to a stirred mixture of the above obtained acetamide and K_2CO_3 (3.766 g, 27.28 mmol, 5 equiv) in anhydrous acetone (37 mL) under nitrogen. The reaction mixture was stirred under reflux for 1 h, allowed to cool to room temperature, filtered through a short plug of celite, washing with acetone, and the filtrate concentrated under reduced pressure. Chromatography of the residue obtained, using hexane-EtOAc mixtures from 100:0 to

50:50, afforded *N*-(4-(benzyloxy)-2,3-dichlorophenyl)acetamide **iii** (1.37 g, 81%). ^1H NMR (300 MHz, CDCl_3) δ 8.18 (1H, d, J = 9.3 Hz, H-5 Ph), 7.43 (6H, m, C2-6 Bn and NH), 6.92 (1H, d, J = 9.3 Hz, H-6 Ph), 5.16 (2H, s, CH_2), 2.23 (3H, s, CH_3).

c) A solution of HCl in dioxane (4M, 12 mL, 46 mmol) was added to a solution of acetamide **iii** (942 mg, 3.036 mmol) in CH_3OH (18.9 mL) and the mixture was refluxed under anhydrous conditions for 2.30 h. The reaction mixture was cooled to room temperature, poured into H_2O and basified to pH 9-10 by the addition of 3M NaOH. The basic mixture was extracted with Et_2O and the organic extracts were washed with brine and dried with anhydrous Na_2SO_4 . Evaporation of the solvent under vacuum left to the solid aniline **4** (745 mg, 95%) whose ^1H NMR spectrum (identical with that previously reported in the literature)¹ showed to be essentially pure and was used without further purification. ^1H NMR (300 MHz, CDCl_3) δ 7.39 (5H, m, C2-6 Bn), 6.77 and 6.61 (each 1H, AB system, J = 9.0 Hz, H-5 and H-6 Ph), 5.06 (2H, s, CH_2), 3.91 (2H, br s, NH_2).

Characterization data of intermediates of the synthesis of hapten FHm

1-Allylcyclohexanecarboxylic acid (2).² IR $\nu_{\text{max}}/\text{cm}^{-1}$ (NaCl) 2500-3000, 1697, 1640, 1453, 1245, 917; ^1H NMR (300 MHz, CDCl_3) δ 11.44 (1H, br s, CO_2H), 5.75 (1H, m, =CH), 5.08 and 5.03 (1H each, each m, = CH_2), 2.30 (2H, d, J = 7.5 Hz, $\text{CH}_2\text{CH=}$), 2.04 (2H, m, H-2/H-6 Cy), 1.59-1.20 (8H, m, 10 CH-Cy); ^{13}C NMR (75 MHz, CDCl_3) δ 183.5 (CO_2H), 133.3 (=CH), 117.9 (=CH₂), 47.1 (C1-Cy), 44.3 ($\text{CH}_2\text{-C=}$), 33.4 (C2/C6-Cy), 25.7 (C4-Cy), 23.0 (C3/C5-Cy).

1-Allyl-N-(4-(benzyloxy)-2,3-dichlorophenyl)cyclohexanecarboxamide (5). IR $\nu_{\text{max}}/\text{cm}^{-1}$ (NaCl) 3431, 3312, 3070, 2930, 2859, 1675, 1592, 1508, 1466, 1272, 1133; ^1H NMR (300 MHz, CDCl_3) δ 8.20 (1H, d, J = 9.3 Hz, H-6 Ph), 7.81 (1H, br s, NH), 7.45-7.29 (5H, m, C2-6 Bn), 6.92 (1H, d, J = 9.3 Hz, H-5 Ph), 5.74 (1H, m, =CH), 5.15 (2H, s, OCH_2), 5.09 and 5.04 (1H each, each m, = CH_2), 2.33 (2H, d, J = 7.5 Hz, $\text{CH}_2\text{CH=}$), 2.05

¹ a) Dinsmore, C. J.; Bergman, J. M. Inhibitors of prenyl-protein transferase. PCT Int. Appl. (2001), WO2001060815 A1, 2001-08-23. b) Practical Application of the Palladium-catalyzed Amination in Phenylpiperazine Synthesis: An Efficient Synthesis of a Metabolite of the Antipsychotic Agent Aripiprazole. *Tetrahedron*, **1998**, *54*, 4811-4818.

² Nicolai, Stefano; Piemontesi, Cyril; Waser, Jerome. A Palladium-Catalyzed Aminoalkynylation Strategy towards Bicyclic Heterocycles: Synthesis of ($\pm$)-Trachelanthamidine. *Angew. Chem. Int. Ed.*, **2011**, *50*, 4680-4683.

(2H, m, H-2/H-6 Cy), 1.70-1.25 (8H, m, 8 CH-Cy); ^{13}C NMR (75 MHz, CDCl_3) δ 174.2 (CON), 151.4 (C4-Ph), 136.1 (C1-Bn), 133.1 (=CH), 129.5 (C1-Ph), 128.6 (C3/C5-Bn), 128.1 (C4-Bn), 127.1 (C2/C6-Bn), 123.6 (C2-Ph), 122.3 (C3-Ph), 120.1 (C6-Ph), 118.5 (=CH₂), 112.5 (C5-Ph), 71.4 (OCH₂), 47.8 (C1-Cy), 44.8 (CH₂-C=), 34.0 (C2/C6-Cy), 25.8 (C4-Cy), 22.8 (C3/C5-Cy); HRMS (TOF MS ES+) calcd for $\text{C}_{23}\text{H}_{26}\text{Cl}_2\text{NO}_2$ $[\text{M}+\text{H}]^+$ 418.1341, found 418.1347.

(*E*)-Methyl 6-(1-((4-(benzyloxy)-2,3-dichlorophenyl)carbamoyl)cyclohexyl) hex-4-enoate (**7**). IR $\nu_{\text{max}}/\text{cm}^{-1}$ (NaCl) 3432, 3310, 2929, 1737, 1679, 1594, 1510, 1470, 1272, 1026; ^1H NMR (300 MHz, CDCl_3) δ 8.18 (1H, d, J = 9.3 Hz, H-6 Ph), 7.79 (1H, br s, NH), 7.47-7.32 (5H, m, C2-6 Bn), 6.92 (1H, d, J = 9.3 Hz, H-5 Ph), 5.37 (2H, m, H-4 and H-5), 5.09 (2H, s, OCH₂), 3.55 (3H, s, CO₂CH₃), 2.30 (4H, m, H-2 and H-3), 2.25 (2H, m, H-6), 2.05 (2H, H-2/H6-Cy), 1.70-1.25 (8H, m, 8 CH-Cy); ^{13}C NMR (75 MHz, CDCl_3) δ 174.3 (CON), 173.4 (CO₂), 151.4 (C4-Ph), 136.1 (C1-Bn), 132.3 (C4), 129.5 (C1-Ph), 128.6 (C3/C5-Bn), 128.1 (C4-Bn), 127.1 (C2/C6-Bn), 125.8 (C5), 125.2 (C2-Ph), 123.6 (C3-Ph), 120.1 (C6-Ph), 112.4 (C5-Ph), 71.4 (OCH₂), 51.5 (OCH₃), 48.0 (C1-Cy), 34.0 (C2/C6-Cy), 33.8 (C2), 27.8 (C3), 25.8 (C4-Cy), 22.8 (C3/C5-Cy); HRMS (TOF MS ES+) calcd for $\text{C}_{27}\text{H}_{32}\text{Cl}_2\text{NO}_4$ $[\text{M}+\text{H}]^+$ 504.1708, found 504.1710.

Methyl 6-(1-((2,3-dichloro-4-hydroxyphenyl)carbamoyl)cyclohexyl)hexanoate (**8**). ^1H NMR (300 MHz, CDCl_3) δ 7.95 (1H, d, J = 9 Hz, H-6 Ph), 7.70 (1H, br s, NH), 6.90 (1H, d J = 9 Hz, H-5 Ph), 6.32 (1H, br s, OH), 3.64 (3H, s, CO₂CH₃), 2.27 (2H, t, J = 7.2 Hz, H-2), 2.04 (2H, H-2/H-6 Cy), 1.70-1.20 (16H, m, H-3, H-4, H-5, H-6 and 8 CH-Cy); ^{13}C NMR (75 MHz, CDCl_3) δ 175.2 (CON), 174.3 (CO₂H), 149.4 (C4-Ph), 128.5 (C1-Ph), 123.4 (C2-Ph), 122.2 (C3-Ph), 119.1 (C6-Ph), 114.6 (C5-Ph), 51.6 (OCH₃), 47.5 (C1-Cy), 40.7 (C6), 34.4 (C2/C6-Cy), 33.9 (C2), 29.5 (C4), 25.9 (C4-Cy), 24.7 (C3), 23.6 (C5), 22.9 (C3/C5-Cy).

Characterization data of intermediates of the synthesis of haptens FHd and FHh

Ethyl 8-methyl-1,4-dioxaspiro[4.5]decane-8-carboxylate (11). IR $\nu_{\max}/\text{cm}^{-1}$ (NaCl) 2953, 2877, 1725, 1446, 1361, 1311, 1203, 1118, 1034, 924; ^1H NMR (300 MHz, CDCl_3) δ 4.09 (2H, q, $J = 7.1$ Hz, OCH_2CH_3), 3.86 (4H, s, $\text{OCH}_2\text{CH}_2\text{O}$), 2.07 (2H, m, H-7/H-9), 1.67-1.42 (6H, m), 1.19 (3H, t, $J = 7.1$ Hz, OCH_2CH_3), 1.22 (3H, s, CH_3); ^{13}C NMR (75 MHz, CDCl_3) δ 176.8 (COO), 108.3 (C5), 64.1 ($\text{OCH}_2\text{CH}_2\text{O}$), 60.2 (OCH_2CH_3), 42.2 (C8), 32.7 (C6/C10), 31.9 (C7/C9), 25.9 (CH_3), 14.1 (OCH_2CH_3); HRMS (TOF MS ES+) calcd for $\text{C}_{12}\text{H}_{21}\text{O}_4$ $[\text{M}+\text{H}]^+$ 229.1440, found 229.1442.

8-Methyl-1,4-dioxaspiro[4.5]decane-8-carboxylic acid (12). IR $\nu_{\max}/\text{cm}^{-1}$ (KBr) 3500-2500, 2952, 2880, 1729, 1698, 1467, 1453, 1244, 1119, 1089, 1038; ^1H NMR (300 MHz, CDCl_3) δ 3.93 (4H, s, $\text{OCH}_2\text{CH}_2\text{O}$), 2.13 (2H, m, H-7/H-9), 1.69-1.65 (4H, m), 1.57-1.31 (m, 2H), 1.25 (3H, s, CH_3); ^{13}C NMR (75 MHz, CDCl_3) δ 183.7 (COOH), 108.4 (C5), 64.2 ($\text{OCH}_2\text{CH}_2\text{O}$), 42.2 (C8), 32.6 (C6/C10), 31.9 (C7/C9), 25.8 (CH_3). HRMS (TOF MS ES+) calcd for $\text{C}_{10}\text{H}_{20}\text{NaO}_4$ $[\text{M}+\text{Na}]^+$ 223.0946, found 223.0947.

N-(4-(Benzyloxy)-2,3-dichlorophenyl)-8-methyl-1,4-dioxaspiro[4.5]decane-8-carboxamide (13). IR $\nu_{\max}/\text{cm}^{-1}$ (NaCl) 3432, 3306, 2951, 2880, 1662, 1596, 1509, 1274, 1116, 1034; ^1H NMR (300 MHz, CDCl_3) δ 8.12 (1H, d, $J = 9$ Hz, H-6 Ph), 7.85 (1H, br s, NH), 7.43-7.28 (5H, m, C2-6 Bn), 6.90 (1H, d, $J = 9$ Hz, H-5 Ph), 5.13 (2H, s, OCH_2), 3.93 ($\text{OCH}_2\text{CH}_2\text{O}$), 2.15 (2H, H-7/H-9), 1.72 (m, 6H), 1.31 (s, CH_3); ^{13}C NMR (75 MHz, CDCl_3) δ 174.9 (CON), 151.5 (C4-Ph), 136.1 (C1-Bn), 129.4 (C1 Ph), 128.6 (C3/C5-Bn), 128.1 (C4-Bn), 127.1 (C2/C6-Bn), 123.8 (C2-Ph), 122.3 (C3-Ph), 120.2 (C6-Ph), 112.4 (C5-Ph), 108.1 (C5), 71.4 (OCH_2), 64.2 ($\text{OCH}_2\text{CH}_2\text{O}$), 43.2 (C8), 33.1 (C6/C10), 31.7 (C7/C9), 21.0 (CH_3); HRMS (TOF MS ES+) calcd for $\text{C}_{23}\text{H}_{26}\text{Cl}_2\text{NO}_4$ $[\text{M}+\text{H}]^+$ 450.1239, found 450.1231.

N-(4-(Benzyloxy)-2,3-dichlorophenyl)-1-methyl-4-oxocyclohexanecarboxamide (**14**). Mp 160-161 °C (from Et₂O-hexane). IR $\nu_{\text{max}}/\text{cm}^{-1}$ (KBr) 3231, 3090, 3009, 2948, 2903, 2864, 1709, 1644, 1572, 1510, 1472, 1454, 1289, 1037; ¹H NMR (300 MHz, CDCl₃) δ 8.12 (1H, d, J = 9 Hz, H-6 Ph), 7.87 (1H, br s, NH), 7.45-7.35 (5H, m, C2-6 Bn), 6.93 (1H, d, J = 9 Hz, H-5 Ph), 5.17 (2H, s, OCH₂), 2.55 (2H, m, H-3/H-5), 2.41 (4H, m, H'-3/H'-5 and H-2/H-6), 1.85 (2H, m, H'-2/H'-6), 1.45 (s, CH₃); ¹³C NMR (75 MHz, CDCl₃) δ 210.1 (C4), 173.9 (CON), 151.9 (C4-Ph), 136.0 (C1-Bn), 128.9 (C1 Ph), 128.6 (C3/C5-Bn), 128.2 (C4-Bn), 127.1 (C2/C6-Bn), 124.2 (C2-Ph), 122.5 (C3-Ph), 120.5 (C6-Ph), 112.4 (C5-Ph), 71.4 (OCH₂), 43.2 (C1), 38.1 (C3/C5), 35.4 (C2/C6), 26.1 (CH₃); HRMS (TOF MS ES+) calcd for C₂₁H₂₂Cl₂NO₃ [M+H]⁺ 406.0981, found 406.0981.

5-(4-((4-(Benzyloxy)-2,3-dichlorophenyl)carbamoyl)-4-methylcyclohexylidene)pentanoic acid (**16**). IR $\nu_{\text{max}}/\text{cm}^{-1}$ (NaCl) 3500-2400, 3430, 3033, 2928, 1707, 1593, 1509, 1469, 1273; ¹H NMR (300 MHz, CDCl₃) δ 8.18 (1H, d, J = 9.2 Hz, H-6 Ph), 7.87 (1H, br s, NH), 7.48-7.29 (5H, m, C2-6 Bn), 6.92 (1H, d, J = 9.2 Hz, H-5 Ph), 5.15 (2H, s, OCH₂), 5.13 (1H, t, J = 7.4, H-5), 2.36 (1H, m, 1H Cy), 2.34 (2H, t, J = 7.4 Hz, H-2), 2.23-2.06 (5H, m, 5H Cy), 2.06 (2H, dt, J = 7.2, 7.2 Hz, H-4), 1.68 (2H, quint, J = 7.3 Hz, H-3), 1.51 (2H, m, 2H Cy), 1.32 (3H, s, CH₃); ¹³C NMR (75 MHz, CDCl₃) δ 179.1 (COOH), 175.3 (CON), 151.5 (C4-Ph), 138.2 (C1-Cy), 136.0 (C1-Bn), 129.4 (C1-Ph), 128.6 (C3/C5-Bn), 128.1 (C4-Bn), 127.1 (C2/C6-Bn), 123.8 (C2-Ph), 122.3 (C3-Ph), 121.3 (C-5), 120.2 (C6-Ph), 112.4 (C5-Ph), 71.4 (OCH₂), 44.4 (C4-Cy), 37.0 (C3-Cy), 36.2 (C5-Cy), 33.3 (C2), 32.9 (C2-Cy), 26.4 (C-4), 26.1 (CH₃), 24.8 (C3), 24.7 (C6-Cy); HRMS (TOF MS ES+) calcd for C₂₆H₂₉Cl₂NNaO₄ [M+Na]⁺ 512.1371, found 512.1375.

Hapten activation and conjugation

The hapten (1 equiv) and DSC (1.1 equiv) were dissolved in anhydrous acetonitrile (1 mL per 0.1 mmol of hapten) under nitrogen in an ice-water bath. Et₃N (3.0 equiv) was then added and the resulting mixture was stirred at 0 °C until complete consumption of the starting material (*ca.* 1–2 h, TLC using CHCl₃/MeOH 9:1 as eluent). The reaction mixture was diluted with CHCl₃, washed with brine, and dried over anhydrous Na₂SO₄. The residue that was obtained after evaporation of the solvent was purified by chromatography, eluting with CHCl₃/MeOH mixtures from 100:0 to 95:5, to give the corresponding NHS esters in moderate yields.

FH*m*-NHS ester: ¹H NMR (300 MHz, CDCl₃) δ 8.10 (1H, d, *J* = 9 Hz, H-6 Ph), 7.72 (1H, br s, NH), 6.96 (1H, d, *J* = 9 Hz, H-5 Ph), 5.64 (1H, br s, OH), 2.81 (br s, 4H, COCH₂CH₂CO), 2.57 (2H, t, *J* = 7.2 Hz, H-2), 2.05 (2H, m, H-2/H-6 Cy), 1.75–1.15 (16H, m).

FH*d*-NHS ester: ¹H NMR (300 MHz, CDCl₃) δ 8.09 (1H, d, *J* = 9.3 Hz, H-6 Ph), 7.76 (1H, br s, NH), 6.96 (1H, d, *J* = 9.3 Hz, H-5 Ph), 5.67 (1H, br s, OH), 5.12 (1H, t, *J* = 7.5, H-5), 2.84 (br s, 4H, COCH₂CH₂CO), 2.60 (2H, t, *J* = 7.5 Hz, H-2), 2.37 (1H, m, 1H Cy), 2.22–2.10 (7H, m, 5H Cy and H-4), 1.79 (2H, quint, *J* = 7.5 Hz, H-3), 1.54 (2H, m, 2H Cy), 1.32 (3H, s, CH₃).

FH*h*-NHS ester: A *ca.* 60:40 mixture of epimers was obtained. ¹H NMR (300 MHz, CDCl₃) (only signals of one of the epimers are given) δ 8.07 (1H, d, *J* = 9 Hz, H-6 Ph), 7.74 (1H, br s, NH), 6.93 (1H, d, *J* = 9.1 Hz, H-5 Ph), 5.91 (1H, br s, OH), 2.84 (br s, 4H, COCH₂CH₂CO), 2.62 (2H, t, *J* = 7.2 Hz, H-2), 2.22 (2H, m, H-3/H-5 Cy), 1.80–1.65 (6H, m, H-3, H-5 and 2H Cy), 1.5–1.03 (7H, m, 7H Cy), 1.31 (3H, s, CH₃).

The purified active esters of the haptens were dissolved in dry *N,N*-dimethylformamide and slowly added to the corresponding protein solution in 50 mM carbonate–bicarbonate buffer, pH 9.6. BSA, OVA, and HRP were used as carriers for immunogen, coating conjugate, and enzyme tracer preparation, respectively. For BSA coupling, 30 μmoles of pure active ester were applied per μmole of carrier protein, whereas for OVA conjugation 3 μmoles of activated hapten were used per mole of protein. Enzyme tracers were obtained by adding a 10-fold molar excess of pure activated hapten to the HRP solution. Conjugation was carried out at rt during 2 h with gentle stirring, and bioconjugates were purified by size exclusion chromatography using desalting columns and 100 mM phosphate buffer, pH 7.4, as eluent.

MALDI-TOF spectra of bioconjugates

Figure S1. MALDI-TOF spectra of a) BSA conjugates, b) OVA conjugates, and c) HRP conjugates of novel fenhexamid haptens. (Reference proteins are depicted in blue line, FHm conjugates in green line, FHd conjugates in orange line and FHH conjugates in cyan line).

Monoclonal antibody generation

Immunization. BALB/c female mice (8–10 weeks old) were immunized with the BSA conjugates by intraperitoneal injections. Doses consisted of an emulsion of 100 μL of PB containing 100 μg of protein conjugate and 100 μL of Freund's adjuvant. The first dose contained complete Freund's adjuvant, and subsequent doses were given at weeks 3 and 6 using incomplete Freund's adjuvant. After a resting period of at least 3 weeks from the last injection with adjuvant and four days before cell fusion, mice received a booster intraperitoneal injection of 200 μL of a 1:1 mixture of PBS and a solution containing 100 μg of BSA-hapten conjugate in PB.

Cell fusion and culture. P3-X63/Ag 8.653 murine myeloma cells were cultured in high-glucose DMEM supplemented with 2 mM alanylglutamine, 1 mM MEM nonessential amino acids, and 25 $\mu\text{g}/\text{mL}$ gentamycin (referred to as s-DMEM) and containing 10% (v/v) foetal bovine serum (FBS). Just before spleen extraction, mouse blood was collected by heart puncture and the serum was diluted 1/10 with storage buffer and kept at 4 $^{\circ}\text{C}$. After cytolysis of red blood cells by osmotic shock, mouse spleen lymphocytes were fused with myeloma cells at a 4:1 ratio using 1 mL of PEG 1500 as the fusing agent. The fused cells were distributed in 96-well culture plates at a density between 1.5×10^5 and 2.5×10^5 cells per well in 100 μL of s-DMEM with 15% FBS. Sixteen hours after plating, 100 μL of HAT selection medium was added to each well.

Hybridoma selection and cloning. Twelve days after fusion, hybridoma culture supernatants were screened by differential indirect cELISA with 1.0 $\mu\text{g}/\text{mL}$ homologous OVA coating conjugate. RAM-HRP conjugate from Dako (Glostrup, Denmark) was employed to detect the immunological reaction. The signal of each blank assay was compared with the corresponding competitive result when 0.1 μM

fenhexamid was used as competitor. Supernatants affording a high ratio between the absorbance of both assays or with saturated signals were rescreened by checkerboard indirect cELISA. Homologous conjugate-coated plates (at 0.1 and 1.0 µg/mL) were employed and serial supernatant and fenhexamid dilutions were combined and assayed. Detected high-affinity antibody-producing hybridomas were cloned by limiting dilution in HT medium containing 20% FBS and 1% HFCS. Stable clones were expanded and cryopreserved in liquid nitrogen.

Table S1
Immunoassay conditions and standard curve parameters for fenhexamid determination

mAb	[mAb] (µg/L)	Tracer	[T] ^a (µg/L)	A _{max}	IC ₅₀ (µg/L)
FHm#110	1000	HRP-FHm	30	1.63	0.14
	1000	HRP-FHd	30	1.48	0.14
	1000	HRP-FHh	30	1.59	0.16
	1000	HRP-FHo	100	--- ^b	---
FHm#113	1000	HRP-FHm	10	0.86	0.05
	1000	HRP-FHd	10	1.12	0.07
	1000	HRP-FHh	10	1.00	0.09
	1000	HRP-FHo	100	--- ^c	---
FHm#114	1000	HRP-FHm	100	1.02	0.02
	1000	HRP-FHd	100	0.61	0.05
	1000	HRP-FHh	30	1.33	0.02
	1000	HRP-FHo	100	---	---
FHd#19	1000	HRP-FHm	10	1.42	0.39
	1000	HRP-FHd	3	0.82	0.60
	1000	HRP-FHh	10	1.08	0.52
	1000	HRP-FHo	100	---	---
FHd#132	1000	HRP-FHm	100	0.71	0.08
	1000	HRP-FHd	30	1.27	0.08
	1000	HRP-FHh	30	0.95	0.18
	1000	HRP-FHo	100	---	---
FHd#139	1000	HRP-FHm	30	1.85	0.10
	1000	HRP-FHd	10	1.56	0.12
	1000	HRP-FHh	30	1.89	0.17
	1000	HRP-FHo	100	---	---
FHh#15	1000	HRP-FHm	100	1.21	0.04
	1000	HRP-FHd	100	0.79	0.10
	1000	HRP-FHh	30	1.19	0.04
	1000	HRP-FHo	100	---	---
FHh#122	1000	HRP-FHm	100	---	---
	1000	HRP-FHd	100	0.57	0.18
	1000	HRP-FHh	100	0.92	0.08
	1000	HRP-FHo	100	---	---
FHh#130	1000	HRP-FHm	100	0.52	0.11
	1000	HRP-FHd	100	---	---
	1000	HRP-FHh	30	0.85	0.08
	1000	HRP-FHo	100	---	---
FHo#22	1000	HRP-FHm	100	---	---
	1000	HRP-FHd	100	---	---
	1000	HRP-FHh	100	---	---
	1000	HRP-FHo	30	1.92	0.93
FHo#26	1000	HRP-FHm	100	---	---
	1000	HRP-FHd	100	---	---
	1000	HRP-FHh	100	---	---
	1000	HRP-FHo	10	1.29	0.66
FHo#27	1000	HRP-FHm	100	---	---
	1000	HRP-FHd	100	---	---
	1000	HRP-FHh	100	---	---
	1000	HRP-FHo	10	1.55	0.69

^a Enzyme tracer concentration. ^b A_{max} value was lower than 0.5.

Table S2
Precision of the standard curve

Parameter	RSD (%)	
	Intra-day ^a	Inter-day ^b
A _{max}	4.2	5.5
Slope	6.9	9.5
IC ₅₀	6.5	5.5

^a n = 5. ^b n = 3.

Figure S2. Matrix interferences with diluted tomato and cucumber QuEChERS extracts over the studied cELISA.

Table S3
Determination of fenhexamid residues in tomato (T) and cucumber (C) samples by the developed cELISA and a reference chromatographic method.

Type	Sample	[Fenhexamid] (μg/kg ± s, n=3)	
		cELISA	UPLC–MS/MS
In-field treated	T1	580 ± 60	650 ± 80
	T2	490 ± 70	510 ± 80
	T3	190 ± 30	180 ± 30
	T4	430 ± 70	480 ± 90
	T5	330 ± 30	310 ± 70
	C1	870 ± 30	700 ± 100
	C2	1000 ± 300	1000 ± 100
	C3	110 ± 30	70 ± 10
	C4	150 ± 20	100 ± 10
	C5	112 ± 20	28 ± 5
Blind spiked	T6	19 ± 4	21 ± 1
	T7	70 ± 9	81 ± 2
	T8	1400 ± 300	1300 ± 100
	T9	900 ± 100	800 ± 100
	T10	700 ± 100	560 ± 7
	T11	290 ± 20	290 ± 40
	T12	210 ± 30	190 ± 20
	T13	110 ± 20	120 ± 20
	C6	100 ± 20	100 ± 3
	C7	1100 ± 100	1000 ± 100
	C8	800 ± 100	680 ± 40
	C9	90 ± 10	110 ± 20
	C10	24 ± 2	24 ± 2
	C11	71 ± 8	75 ± 2
	C12	280 ± 30	250 ± 30
	C13	1300 ± 100	1100 ± 100

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¹H NMR spectra of haptens FH*m*, FH*d* and FH*h* and their NHS-esters

¹H NMR of hapten FHm (CD₃OD, 300 MHz)

¹H NMR of FHm-NHS ester (CDCl₃, 300 MHz)

^1H NMR of hapten FHd (CDCl₃, 300 MHz)

¹H NMR of FHd-NHS ester (CDCl₃, 300 MHz)

¹H NMR of hapten FHh (CDCl₃, 300 MHz)

¹H NMR of FHh-NHS ester (CDCl₃, 300 MHz)