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Production and Characterization of Monoclonal and Polyclonal Antibodies to Forchlorfenuron

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The development of immunoassays for the detection of the plant growth regulator forchlorfenuron (CPPU) is described. To achieve that purpose, a set of CPPU derivatives has been obtained by the previous synthesis of the adequate *p*-aminophenyl alkanoic acid. Protein conjugates of these compounds have been used as immunogens to produce rabbit polyclonal antibodies and a collection of mouse monoclonal antibodies. Additionally, a battery of structural analogues of the target analyte has been synthesized and used for the characterization of antibody binding. This strategy has demonstrated that most antibodies followed Landsteiner's principle, although some monoclonal antibodies showing important deviations from this behavior have also been found. Finally, different assay formats have been developed with a variety of antibodies and conjugates, and a rapid procedure has been optimized for the indirect ELISA format using monoclonal and polyclonal antibodies. In the indirect competitive ELISA, assay IC_{50} values for CPPU below 0.5 nM were found with LODs as low as 0.013 nM.

KEYWORDS: CPPU; growth promoter; plant growth regulator; phytohormones; cytokinin-like; phenylurea; ELISA; immunoassay; binding site; cross-reactivity

INTRODUCTION

Forchlorfenuron, or *N*-phenyl-*N*′-(2-chloro-4-pyridyl)urea (CPPU), was first synthesized in Japan in 1978 among other compounds that were obtained by inserting electron-withdrawing substituents into the pyridyl ring of *N*-phenyl-*N*′-(4-pyridyl)urea (PPU), creating a phenylurea derivative with strikingly strong cytokinin-like activity in the tobacco callus bioassay (*1*). Thereafter, CPPU was known as a synthetic phytohormone that has a physiological activity on many plants greater than that of the naturally ocurring adenine-substituted cytokinins, such as *trans*-zeatin (**Figure 1**). The mechanism of action of phenylurea derivatives remains unclear, but it has been shown that CPPU displays a cytokinin-like activity 10000 times higher than that of diphenylurea (dPU) and 10 times higher than that of kinetin—a synthetic plant growth regulator analogous to the endogenous hormone (*2, 3*). By the late 1980s, several papers had been published demonstrating the strong commercial potential of CPPU on a variety of crop plants, particularly kiwifruit and table grapes (*4*−*7*). In addition, recent studies have shown that cytokinins, especially CPPU, effectively improve fruit set and fruit enlargement in many crops such as grape, kiwifruit, gourd, watermelon, cucumber, avocado, apple, and pear (*8, 9*). CPPU was first registered for use on table grapes in South Africa, Chile, and Mexico in the mid-1990s. Then, this chemical became commercially available in the United States for table grape and kiwifruit markets (*10*), and recently, it has been included in Annex I of the European Commission Directive 91/414/EEC as an authorized plant growth regulator for kiwifruit (*11*). Nowadays, CPPU is used to increase berry size and quality, to improve pack-out, and to reduce berry shatter and fruit abortion. It has a low order of toxicity both to plants and to animal systems, with an acute dermal toxicity in rabbits of

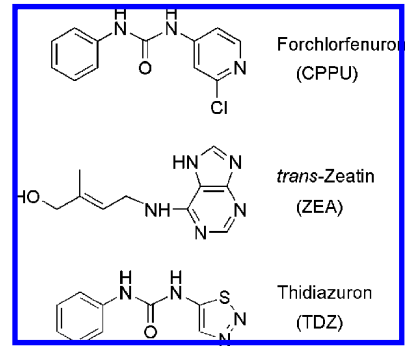

Figure 1. Chemical synthesis of forchlorfenuron, a natural cytokinin *trans*-zeatin, and the herbicide thidiazuron.

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>2000 mg/kg (*12*). During the present decade, a few papers have been published concerning the development of analytical methods based on separation techniques, mainly HPLC, for the detection of CPPU in foods (*13, 14*).

Up to the present study, no antibodies have previously been reported against CPPU, and hence, no immunoassays are available for the analysis of this agrochemical. Despite its low toxicity, the presence of residues of CPPU in food is regulated in most countries. Therefore, the availability of a simple, rapid, and cheap assay for this molecule might be of great significance, not only for public and private quality control laboratories but also for research laboratories devoted to plant physiology studies. Immunoassays constitute a very versatile technique that may be adapted to different assay formats for a variety of applications, which has made them a very valuable complementary tool to the traditional chromatographic methods. The enzyme-linked immunosorbent assay (ELISA) is probably the most well-known type of immunoassay, both in food diagnostics and in research laboratories. To fulfill the specific analytical demands of a certain application for the analysis of a small organic molecule such as CPPU, two classes of immunoreagents are required, that is, a high-affinity antibody (to easily eliminate possible matrix effects by direct dilution) and at least two conjugates of the analyte, one for the immunization of animals and one with a different carrier protein for the competitive assays. Once the immunizing conjugate has been prepared, the question arises whether to obtain polyclonal or monoclonal antibodies (pAbs and mAbs, respectively). If an unlimited supply of a single and homogeneous type of immunoreagent is required, then the choice is monoclonal technology. Nevertheless, high-affinity pAbs against small organic compounds are usually obtained with little trouble. Furthermore, it is not clear whether pAbs or mAbs are the best option regarding selectivity. In the present study, derivatives of CPPU have been synthesized and high-affinity pAbs and mAbs have been generated. The affinity, selectivity, and performance of these antibodies have been compared in homologous and heterologous ELISAs. Also, recognition studies were conducted with the whole set of antibodies using several structural analogues, some of them intentionally synthesized for this purpose. Finally, enzyme immunoassays in different formats have been investigated: the conjugate-coated indirect competitive ELISA (ic-ELISA) by a two-step and a one-step procedure and the antibody-coated direct competitive ELISA (dc-ELISA).

MATERIALS AND METHODS

Chemicals and Instrumentation. Standard forchlorfenuron [1-(2-chloro-4-pyridyl)-3-phenylurea, CPPU] (CAS Registry No. 68157-60-8, MW 247.7 g/mol), thidiazuron (TDZ), 1,3-diphenylurea (dPU), 1-(3-chlorophenyl)-3-phenylurea (CdPU), 1-(3-nitrophenyl)-3-phenylurea (NdPU), and 1-(4-pyridyl)-3-phenylurea (PPU) were acquired from Sigma-Aldrich (Madrid, Spain). Tributylamine (TBA), 6-benzylaminopurine (BAP), 6-furfurylaminopurine (kinetin, KIN), and 6-(4-hydroxy-3-methylbut-2-enylamino)purine (*trans*-zeatin, ZEA) were from Fluka (Madrid, Spain). Stock solutions were prepared in dried *N*,*N*-dimethylformamide (DMF) and stored at −20 °C. 2-(4-Aminophenyl)acetic acid (**4a**) and all other reagents used for the synthesis of the haptens and analogous competitors were acquired from commercial sources and used without further purification. All solvents were purified by distillation and, if required, they were dried according to standard methods. The reactions were monitored with the aid of thin-layer chromatography (TLC) using 0.25 mm precoated silica gel plates. Visualization was carried out with UV light and aqueous ceric ammonium molybdate solution. 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). Preparative reverse-phase HPLC separations were performed with a Waters chromatograph equipped with a Tracer Excel 120 ODS-B C18 column (15 × 1 cm, 5 μm; Teknokroma, Barcelona, Spain), using the eluent indicated in each case. All melting points were determined using a Kofler hot-stage apparatus or a Büchi melting point apparatus and are uncorrected. All NMR spectra were recorded in $CDCl_3$ or DMSO-d_6 at room temperature on a Bruker AC-300 spectrometer (300.13 MHz for ^{1}H and 75.47 MHz for ^{13}C). The spectra were referenced to residual solvent protons in the ^{1}H NMR spectra (7.26 and 2.50 ppm) and to solvent carbons in the ^{13}C NMR spectra (77.0 and 39.43 ppm). Carbon substitution degrees were established by distortionless enhancement by polarization transfer pulse sequences. A combination of correlation spectroscopy and heteronuclear single quantum coherence experiments was utilized for the assignment of ^{1}H and ^{13}C chemical shifts. Infrared (IR) spectra were measured as thin films between NaCl plates for liquid compounds and as KBr pellets for solids using a Nicolet Avatar 320 spectrometer. Electron-impact (EI) and fast atom bombardment (FAB) mass spectra (MS and HRMS) were obtained with a Micromass VG Autospec spectrometer. All operations involving air-sensitive reagents were performed under an inert atmosphere of dry argon using syringes and oven-dried glassware. Although most of the compounds used in this work present minor or usual safety concerns, it is recommended that special precautions for the handling of the phenylisocyanates and isocyanatopyridines be taken because they are skin and eye irritants and harmful if swallowed or inhaled.

Sephadex G-25 HiTrap Desalting columns and HiTrap Protein G HP columns from General Electric Healthcare (Uppsala, Sweden) were used for conjugate and antibody purification, respectively. Polyclonal rabbit anti-mouse immunoglobulin peroxidase conjugate (RAM–HRP) was from Dako (Glostrup, Denmark), and polyclonal goat anti-rabbit immunoglobulin peroxidase conjugate (GAR–HRP) was from Bio-Rad (Madrid, Spain). Reagents for protein conjugation and immunoassays were from Sigma (Madrid, Spain). Cell-biology media and reagents were acquired from regular suppliers. Culture plastic ware and Costar flat-bottom high-binding polystyrene ELISA plates were from Corning (Corning, NY). Ultraviolet−visible (UV−vis) spectra and ELISA absorbances were read (in dual wavelength mode, 492−650 nm) with a PowerWave HT from BioTek Instruments (Winooski, VT). ELISA plates were washed with an ELx405 microplate washer also from BioTek Instruments.

Buffers, Media, and Solutions: (1) PB, 100 mM sodium phosphate buffer, pH 7.4; (2) PBS, 10 mM phosphate buffer, pH 7.4, with 137 mM NaCl and 2.7 mM KCl; (3) PBST, PBS containing 0.05% (v/v) Tween 20; (4) coating buffer, 50 mM carbonate−bicarbonate buffer, pH 9.6; (5) washing solution, 0.15 M NaCl containing 0.05% (v/v) Tween 20; (6) developing buffer, 2 mg/mL of *o*-phenylenediamine and 0.012% (v/v) H_2O_2 in 25 mM citrate and 62 mM sodium phosphate buffer, pH 5.4; (7) stop solution, 2.5 M H_2SO_4.

Hapten Synthesis. Preparation of the CPPU derivatives (p2 and p6, **Figure 2**) was effected by reaction of 2-chloro-4-isocyanatopyridine (**3**), prepared from commercially available 4-carboxypyridine 1-oxide [**1**, (*15*)], with the appropriate 4-aminophenyl alkanoic acid **4**. 6-(4-Aminophenyl)hexanoic acid (**4b**) was prepared from 1-iodo-4-nitrobenzene as described in the Supporting Information. Heterologous haptens CdPUp6 and PPUp6 (**Figure 2**) were synthesized following a similar procedure using 3-chlorophenyl isocyanate (**6**) and freshly prepared azido(pyridine-4-yl)methanone (**9**), respectively.

*Preparation of 2-(4-(3-(2-Chloropyridin-4-yl)ureido)phenyl)acetic Acid (***5a***, Hapten p2).* A solution of 2-chloroisonicotinoyl azide (**2**, 481.0 mg, 2.64 mmol) in toluene (1.0 mL) was added dropwise to a stirred solution of 2-(4-aminophenyl)acetic acid (**4a**, 359.0 mg, 2.38 mmol) in dry toluene (1.2 mL) at reflux under argon. The reaction mixture was stirred for an additional 1.5 h at reflux and then cooled to room temperature. The product was obtained by precipitation from the reaction mixture and washing with toluene and ethyl ether, to give pure hapten p2 (**5a**, 698.0 mg, 96%) as a white solid: mp 238 °C (from THF/MeOH); ^{1}H NMR (DMSO-d_6), δ 12.24 (1H, br s, OH), 9.31 and 8.92 (1H each, each s, two NH), 8.13 (1H, d, J = 5.7 Hz, H-6 Py), 7.61 (1H, d, J = 1.8 Hz, H-3 Py), 7.35 (2H, d, J = 8.5 Hz, H-3/H-5 Ph), 7.27 (1H, dd, J = 5.6, 1.9 Hz, H-5 Py), 7.14 (2H, d, J = 8.5 Hz, H-2/H-6 Ph), 3.46 (2H, s, H-2); ^{13}C NMR (DMSO-d_6), δ 172.76 (C-

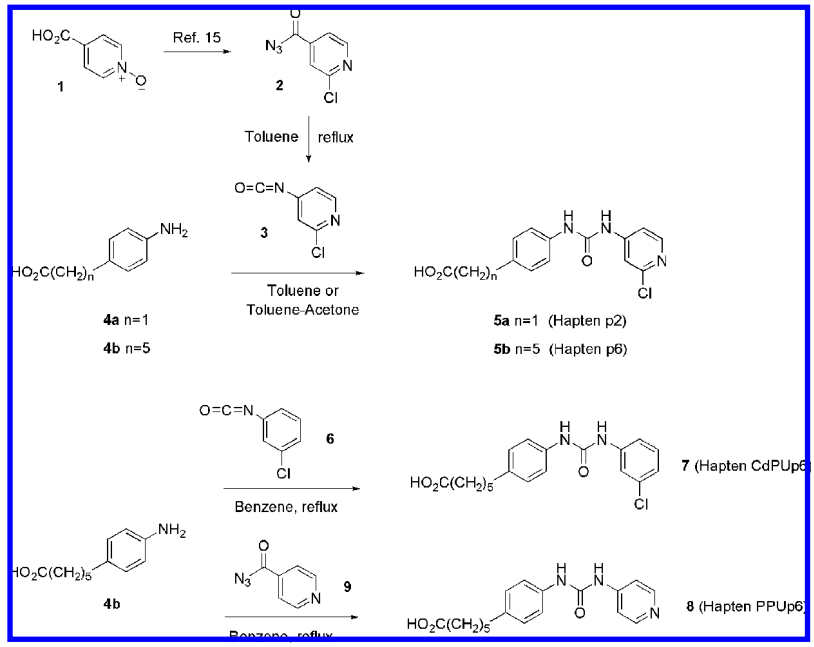

Figure 2. Strategy for the synthesis of the haptens used to prepare the immunogens and the assay conjugates.

1), 151.79 (NCON), 150.93 (C-4 Py), 149.80 (C-6 Py), 149.11 (C-2 Py), 137.28 (C-4 Ph), 129.68 (C-3/C-5 Ph), 129.19 (C-1 Ph), 118.64 (C2/C6 Ph), 111.77 (C-3 Py), 111.19 (C-5 Py), 40.02 (C-2); IR (KBr), 3361, 1730, 1675, 1585, 1509, 1186, 1004, 807 cm^{-1}; MS (EI), *m/z* (%) 305 (M^+, 1), 261 (1), 178 (1), 177 (8), 156 (10), 154 (32), 151 (32), 132 (23), 128 (26), 106 (100); HRMS, calcd for $C_{14}H_{12}ClN_3O_3$ 305.05672, found 305.05443; UV (PB), ε (280 nm) = 16.42 mM^{-1} cm^{-1}, ε (260 nm) = 32.63 mM^{-1} cm^{-1}.

*Preparation of 6-(4-(3-(2-Chloropyridin-4-yl)ureido)phenyl)hexanoic Acid (***5b***, Hapten p6).* A solution of isocyanate **3**, prepared from **2** (88.0 mg, 0.48 mmol) as mentioned before, in toluene (0.8 mL) was added dropwise via syringe to a stirred solution of 6-(4-aminophenyl)-hexanoic acid (**4b**, 90.0 mg, 0.44 mmol) in anhydrous acetone (2.5 mL). The mixture was stirred at room temperature for approximately 3 h and then evaporated to dryness to give a solid residue, which was washed several times with toluene, affording almost pure hapten p6 (**5b**, 115.0 mg, 73% yield) as a white solid. An analytical sample was obtained by preparative reverse phase HPLC using MeOH/H_2O 8:2 as eluent: mp 180−183 °C (from AcOEt); ^{1}H NMR (DMSO-d_6), δ 12.00 (1H, br s, OH), 9.64 and 9.17 (1H each, each s, two NH), 8.17 (1H, d, J = 5.6 Hz, H-6 Py), 7.67 (1H, d, J = 1.8 Hz, H-3 Py), 7.38 (2H, d, J = 8.3 Hz, H-3/H-5 Ph), 7.36 (1H, m overlapped with d at 7.38, H-5 Py), 7.13 (2H, d, J = 8.3 Hz, H-2/H-6 Ph), 2.53 (2H, t, J = 7.5 Hz, H-6), 2.19 (2H, t, J = 7.2 Hz, H-2), 1.55 (4H, m, H-3 and H-5), 1.29 (2H, m, H-4); ^{13}C NMR (DMSO-d_6), δ 174.50 (C-1), 151.84 (NCON), 150.88 (C-4 Py), 149.88 (C-6 Py), 149.20 (C-2 Py), 136.51 and 136.36 (C-1 and C-4 Ph), 128.54 (C-3/C-5 Ph), 118.81 (C2/C6 Ph), 111.77 (C-3 Py), 111.10 (C-5 Py), 34.31 (C-6), 33.64 (C-2), 30.74 (C-5), 28.12 (C-4), 24.32 (C-3); IR (KBr), 3353, 2930, 2854, 1718, 1585, 1507, 1187, 1004, 841 cm^{-1}; MS (EI), *m/z* (%) 233 (4), 207 (28), 154 (24), 155 (9), 154 (24), 119 (16), 132 (17), 106 (100); HRMS (FAB), calcd for $C_{18}H_{21}ClN_3O_3$ [M^+ + 1] 362.12714, found 362.12816. UV (PB), ε (280 nm) = 13.65 mM^{-1} cm^{-1}, ε (260 nm) = 26.91 mM^{-1} cm^{-1}.

*Preparation of 6-(4-(3-(3-Chlorophenyl)ureido)phenyl)hexanoic Acid (***7***, Hapten CdPUp6).* 3-Chlorophenyl isocyanate (**6**, 30.1 μL, 0.25 mmol) was added dropwise via syringe to a solution of 6-(4-aminophenyl)hexanoic acid (**4b**, 46.9 mg, 0.23 mmol) in anhydrous benzene (1.0 mL) at reflux. The reaction was stirred for 1.5 h at reflux and then cooled to room temperature. The solid was collected by filtration, washed with hexane, and dried under vacuum to afford pure hapten CdPUp6 (59.0 mg, 72% yield) as a white solid: mp 179−182 °C (from DMSO-H_2O); ^{1}H NMR (DMSO-d_6), δ 11.98 (1H, br s, OH), 8.84 and 8.64(1H each, each s, two NH), 7.71 (1H, br t, H-2 ClPh), 7.37−7.22 (4H, m, H-3/H-5 Ph, H-5 and H-6 ClPh), 7.09 (2H, br d, J = 7.1 Hz, H-2/H-6 Ph), 7.00 (1H, br dt, J = 7.2, 1.6 Hz, H-4 ClPh), 2.50 (2H, t overlapped with solvent signal, H-6), 2.19 (2H, t, J = 7.3 Hz, H-2), 1.54 (4H, m, H-3 and H-5), 1.27 (2H, m, H-4); ^{13}C NMR (DMSO-d_6), δ 174.45 (C-1), 152.34 (NCON), 141.32 (C-1 ClPh), 136.95, 135.86, and 133.12 (C-1 Ph, C-4 Ph and C-3 ClPh), 130.30 (C-5 ClPh), 128.48 (C2/C6 Ph), 121.22 (C-2 ClPh), 118.43 (C3/C5 Ph), 117.37 and 116.46 (C-4 and C-6 ClPh), 34.29 and 33.59 (C-2 and C-6), 30.76 (C-5), 28.10 (C-4), 24.30 (C-3); IR (KBr), 3301, 2929, 2846, 1685, 1633, 1586, 1555, 1397, 1306, 924, 845, 790, 774, 545 cm^{-1}; MS (EI), *m/z* (%) 300 (5), 233 (7), 207 (9), 171 (4), 152 (100), 127 (85), 106 (54); HRMS (FAB), calcd for $C_{19}H_{22}ClN_2O_3$ [M^+ + 1] 361.13189, found 361.13072; UV (PB), ε (280 nm) = 7.03 mM^{-1} cm^{-1}, ε (260 nm) = 28.23 mM^{-1} cm^{-1}.

*Preparation of 6-(4-(3-(Pyridine-4-yl)ureido)phenyl)hexanoic Acid (***8***, Hapten PPUp6).* A solution of isonicotinic acid (500.0 mg, 4.06 mmol) in anhydrous 1,4-dioxane (10.0 mL) was treated with triethylamine (850.0 μL, 6.09 mmol) and diphenylphosphoryl azide (1.3 mL, 6.09 mmol). The reaction was stirred at 40 °C for 2 h, and then it was cooled to room temperature and concentrated under vacuum. The solid residue obtained was purified by silica gel chromatography, using CH_3Cl as eluent, to give azido(pyridine-4-yl)methanone (**9**, 430.6 mg, 72% yield) as a slightly orange oil (*16*).

A solution of the above purified acyl azide **9** (118.0 mg, 0.80 mmol) in dry benzene (2.0 mL) was stirred at reflux during 30 min. Then, a solution of 6-(4-aminophenyl)hexanoic acid (**4b**, 100.0 mg, 0.48 mmol) in anhydrous benzene (4.0 mL) was added dropwise via syringe to the solution of the formed isocyanate, and the mixture was stirred for 2.5 h at reflux and then cooled to room temperature. The solid obtained after filtration was purified by column chromatography, using CH_3Cl/MeOH 9:1 as eluent, to give hapten PPUp6 (**8**, 42.2 mg, 27% yield) as a slightly brown solid: mp 192−195 °C (from MeOH); ^{1}H NMR (DMSO-d_6), δ 11.99 (1H, br s, OH), 9.10 and 8.80 (1H each, each s, two NH), 8.34 (2H, d, J = 6.0 Hz, H-2/H-6 Py), 7.42 (2H, d, J = 6.0 Hz, H-3/H-5 Py), 7.35 (2H, d, J = 8.4 Hz, H-3/H-5 Ph), 7.11 (2H, d, J = 8.4 Hz, H-2/H-6 Ph), 2.50 (2H, t overlapped with solvent signal, H-6), 2.19 (2H, t, J = 7.3 Hz, H-2), 1.50 (4H, m, H-3 and H-5), 1.27 (2H, m, H-4); ^{13}C NMR (DMSO-d_6), δ 174.40 (C-1), 165.19 (NCON), 152.03 (C-4 Py), 150.00 (C-2/C-6 Py), 136.62 and 136.18 (C-1 and C-4 Ph), 128.51 (C-2/C-6 Ph), 118.56 (C3/C5 Ph), 112.10 (C-3/C-5 Py), 34.25 and 33.55 (C-2 and C-6), 30.73 (C-5), 28.07 (C-4), 24.27 (C-3); IR (KBr) 3334, 3295, 3251, 2923, 2853, 1719, 1593, 1509, 1309, 1240, 1179, 831 cm^{-1}; MS (EI), *m/z* (%) 304 (1), 262 (3), 233 (7), 208 (2), 207 (14), 178 (5), 171 (1), 161 (2), 132 (23), 120 (41), 106 (100); HRMS (FAB), calcd for $C_{18}H_{22}N_3O_3$ [M^+ + 1] 328.16612, found 328.16608; UV (PB), ε (280 nm) = 7.85 mM^{-1} cm^{-1}, ε (260 nm) = 11.41 mM^{-1} cm^{-1}.

Synthesis of Analogous Competitors. The following ureas, previously known but not commercially available, which introduced modified aromatic moieties with respect to CPPU, were synthesized. 1-(2-Chloro-4-pyridyl)-3-(4-fluorophenyl)urea (4F-CPPU) and 1-(2-chloro-4-pyridyl)-3-(2,3,4,5,6-pentafluorophenyl)urea (PF-CPPU) were prepared as described by Abad et al. (*17*). 1-(2,6-Dichloro-4-pyridyl)-3-phenylurea (dCPPU) was synthesized according to the method of Okamoto et al. (*18*). 1-(2-Chloropyridin-4-yl)-3-(pyridine-4-yl)urea (CdPyU) (*19*), 1-(2-chloropyridin-4-yl)-3-*p*-tolylurea (4M-CPPU), and 1-(2-bromopyridin-4-yl)-3-phenylurea (BPPU) (*20*) were prepared as described in the Supporting Information.

Protein−Hapten Conjugates. All conjugates used in this study were prepared by activation of the free carboxylic group of the hapten and reaction with the amine groups of the carrier protein. Three carrier proteins were used: BSA for the immunizing conjugates, OVA for coating conjugates, and HRP for tracer conjugates. Thus, haptens p2 and p6 were coupled to BSA using the active ester method, whereas OVA and HRP conjugates of these haptens and those of PPUp6 and CdPUp6 were prepared according to the mixed anhydride method. For further details see the Supporting Information.

Production of Antibodies. *Rabbit Polyclonal Antibodies.* Animal manipulation has been performed in compliance with the laws and guidelines of the Spanish Ministry of Agriculture, Fisheries, and Food. Two female New Zealand white rabbits weighing 1−2 kg were immunized by subcutaneous injection with 0.3 mg of BSA−p6 conjugate in a 1 mL of a 1:1 mixture of PB and complete Freund's adjuvant. Animals were boosted at 21-day intervals with the same immunogen suspended in a mixture of 0.5 mL of PB and 0.5 mL of incomplete Freund's adjuvant. Ten days after the third boost, blood was obtained by bleeding the ear vein of the rabbits. Whole blood was collected from the ear vein of the rabbits and by intracardiac puncture 10 days after the fourth injection. Blood samples were allowed to coagulate overnight at 4 °C. Then, the serum was separated by centrifugation, and a fraction was diluted 1/5 with PBS containing 0.01% thimerosal (w/v) and kept at 4 °C for daily usage. The remainder of each antiserum was precipitated with a solution of saturated ammonium sulfate, and the precipitates were stored at 4 °C. The antiserum titer was defined as the reciprocal of the dilution that results in a maximum absorbance value (A_{max}) around 1.0 under the described conditions.

Mouse Monoclonal Antibodies. Eight BALB/c female mice were immunized with the BSA–p2 or BSA–p6 conjugate (four animals with each conjugate) by intraperitoneal injections. The antiserum from each mouse was obtained by submandibular bleeding 9 or 10 days after the third injection. Sera were diluted 1/10 with PBS containing 1% (w/v) BSA and 0.01% (w/v) thimerosal and kept at 4 °C in amber vials. Standard procedures for immunization, cell fusion, hybridoma cloning, and antibody purification are described in the Supporting Information. Cell fusion yield was defined as the percentage number of wells with growing hybridomas. The screening of hybridomas was conducted by simultaneous indirect noncompetitive and competitive ELISA as described by Abad et al. (*21*), with 1.0 or 0.1 μM CPPU as competitor. The immunoglobulin isotype was determined using the ImmunoPure Monoclonal Antibody Isotyping Kit I (HRP/ABTS) from Pierce (Rockford, IL).

Competitive ELISAs. CPPU and all other analytes were prepared as a concentrated solution in DMF and kept at −20 °C in amber glass vials. Sigmoidal curves were mathematically fitted to a four-parameter logistic equation using the SigmaPlot software package from SPSS Inc. (Chicago, IL). The assays consisted of the simultaneous evaluation of different concentrations of both the immobilized immunoreagent and the immunoreagent in solution. First, 96-well polystyrene ELISA plates were coated with 100 μL of hapten conjugate or antibody in coating buffer by overnight incubation at room temperature. The next day, the coated plates were washed four times with washing solution, and then they received 50 μL per well of CPPU or analyte solution serially diluted in PBS plus 50 μL per well of (a) primary antibody solution, (b) primary and secondary antibody mixture, or (c) tracer conjugate in PBST, depending on the format. All samples were run in single wells. The immunological competitive reaction took place during 1 h at room temperature, and then the plates were washed as before. Two-step assays were continued by incubation during 1 h at room temperature with 100 μL per well of a solution of the secondary antibody−enzyme conjugate in PBST, and finally the plates were washed again. The retained peroxidase activity was determined after washing by the addition of 100 μL per well of freshly prepared developing buffer. The enzymatic reaction was stopped after 10 min at room temperature by the addition of 100 μL per well of stop solution. The absorbance was immediately read at 492 nm with a reference wavelength at 650 nm. Assay sensitivity was estimated as the concentration of analyte that reduced 50% (IC_{50}) the maximum signal reached at the zero dose of analyte. The limit of detection (LOD) was estimated as the concentration of CPPU that provided a 10% reduction of A_{max}. Cross-reactivity (CR) values were calculated according to the formula

$$CR = [IC_{50}(CPPU)/IC_{50}(compound)] \times 100 \quad (1)$$

Particular Assay Conditions. (a) For the two-step ic-ELISAs, plates were coated with OVA conjugate solutions at 1.0 and 0.1 μg/mL. The next day, the competitive reaction was performed with rabbit serum, mouse serum, hybridoma supernatant, or immunoglobulin solution in PBST, and a 1/10000 dilution of GAR−HRP conjugate or a 1/2000 dilution of RAM−HRP conjugate was used in the secondary reaction. (b) Regarding the one-step ic-ELISAs, plates were prepared with 1.0 and 0.1 μg/mL of OVA conjugate. Competitive assays were carried out with a 1:1 (v/v) mixture of mAb or pAb and HRP-labeled secondary antibody solutions in PBST. The mixtures were freshly prepared just before use, and they were left for at least 10 min at room temperature before they were added to the coated plates. (c) Finally, for dc-ELISAs, plates were coated with a 1.0 μg/mL solution of mAb or a dilution of the corresponding pAb in coating buffer.

RESULTS AND DISCUSSION

Hapten Synthesis and Conjugate Preparation. The molecule of CPPU comprises two aromatic rings bridged by a polar urea group. Theoretically, any of these moieties constitutes an important immunodeterminant group for antibody generation, but it is the pyridyl ring that distinguishes CPPU from other phenylurea compounds. Optimum spacer lengths probably depend upon the physicochemical properties of each particular hapten. CPPU is a rather symmetric, small-to-medium-sized hapten with hydrophilic and hydrophobic chemical groups. Nowadays, it is still difficult to predict the proper linker length for a certain hapten. Attending to these considerations, the immunizing haptens were prepared by introduction of linear hydrocarbon spacer arms of two different lengths (two and six carbon atoms) at a distal site from the pyridyl moiety, that is, the para position of the phenyl ring (**Figure 2**). Our synthetic strategy resembles previously described approaches to obtain hapten derivatives of other phenylurea pesticides, such as monuron, diuron, and isoproturon (*22*−*24*). The isocyanate **3** is very unstable, so it had to be prepared in situ from the corresponding azide **2** as a one-pot reaction. The final molecule with the spacer arm could easily be produced by employing the appropriate *p*-aminophenyl alkanoic acid. In this respect, compound **4b** is a key intermediate reactant because, unlike **4a**, it is not commercially available. The synthesis of this chemical, originally described by Van der Scheer (*25*), was accomplished in this work by Sonoghasira coupling (see the Supporting Information), a reaction that is routinely used in our laboratory for the preparation of a number of haptens. This reaction makes possible the introduction of an alkane chain to an aromatic ring and therefore the functionalization of target analytes. In addition to the immunizing haptens, two derivatives were synthesized with single differences compared to the molecule of CPPU (**Figure 2**). The synthesis of these heterologous haptens was achieved following a strategy similar to that used to prepare hapten p6. On the one hand, a derivative was prepared in which the chloropyridine was substituted by a chlorophenyl ring, yet

Table 1. Analysis of Mouse Antisera Collected after the Third Injection

		mouse antisera											
		Mp2#1			Mp2#2			Mp2#3			Mp2#4		
OVA conjugate	μg/mL	dil[a]	A_{max}[b]	IC_{50}[c]	dil	A_{max}	IC_{50}	dil	A_{max}	IC_{50}	dil	A_{max}	IC_{50}
p2	0.1	90	0.85	14	30	1.08	9	30	1.59	18	30	1.10	27
	1.0	270	0.86	355	90	1.37	144	90	1.76	840	90	1.35	261
p6	0.1	nd[d]	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
	1.0	90	1.38	25	90	0.80	33	270	0.80	15	90	1.01	33
		mouse antisera											
		Mp6#1			Mp6#2			Mp6#3			Mp6#4		
OVA conjugate	μg/mL	dil	A_{max}	IC_{50}	dil	A_{max}	IC_{50}	dil	A_{max}	IC_{50}	dil	A_{max}	IC_{50}
p2	0.1	nd	nd	nd	50	1.05	64	100	0.97	13	50	1.39	44
	1.0	nd	nd	nd	200	1.46	928	100	2.00	625	100	2.15	2500
p6	0.1	nd	nd	nd	30	0.41	330	nd	nd	nd	10	0.78	125
	1.0	nd	nd	nd	90	1.14	420	80	1.39	31	90	1.24	200

[a] Dilution factors ($\times 10^3$). [b] Values of a single determination. [c] Values in nM for CPPU as competitor. [d] Not determined.

keeping the chlorine atom at the same position (CdPUp6). On the other hand, a hapten without the characteristic chlorine atom but maintaining the pyridyl ring was synthesized (PPUp6). These two heterologous molecules contained the same spacer arm, in both length and composition, which was placed at the same site as the immunizing haptens (p2 and p6). Because of the chemical modifications that had been introduced, it was expected that such compounds might be recognized with a lower affinity than the homologous haptens, which consequently may improve assay sensitivity.

Haptens p2 and p6 were conjugated to BSA using the active ester method to obtain the immunizing conjugates. The absorbance spectra of both conjugates showed an important modification with respect to that of the uncoupled BSA. From the absorbance values at 280 and 260 nm of the conjugates, the estimated final hapten-to-protein molar ratios (MRs) were 24 and 30 for p2 and p6, respectively. All four synthesized haptens were also coupled to OVA and HRP and used as assay conjugates. The final MRs of OVA conjugates were determined from the absorbance values at 280 and 260 nm, and they were 2.8, 2.4, 4.5, and 2.5 for p2, p6, CdPUp6, and PPUp6, respectively. These values enter into the usual range obtained with this procedure, considering the 20 ε-amine residues accessible in OVA. Moreover, the final MRs of HRP conjugates were established from the absorbance values at 280 and 400 nm, and they were 2.1, 3.4, 7.5, and 1.5 for p2, p6, CdPUp6, and PPUp6, respectively. Certainly, the calculated MR for the conjugate HRP−CdPUp6 is too high, exceeding the two Lys residues usually available in this enzyme. An alteration of the extinction coefficients of the hapten and/or the protein due to conjugation, or other experimental artifacts, might be responsible for this overestimated value.

Animal Immunization and Antibody Production. *Rabbit Antisera.* Two rabbits were immunized with the BSA−p6 conjugate. Animal sera were collected after the third and fourth injections. Initial characterization was done by bidimensional competitive assays using the two-step ic-ELISA with serial dilutions of the antisera at 1.0 and 0.1 μg/mL homologous coating conjugate concentrations. Very high titers for both rabbit antisera were already observed after the third immunization, affording assays with IC_{50} values below 10 nM for CPPU with both coating concentrations. An increase of 3−5 times in the titer of the antisera was observed between the third and fourth boosts (results not shown). As an example, the titer of Rp6#1 using 0.1 μg/mL of OVA−p6 coating conjugate was 2×10^5 after the third immunization, and after the fourth immunization, it increased to 1×10^6.

Mouse Antisera. Mice were immunized with a BSA conjugate of either p2 or p6, and animal sera were collected after the third and fourth injections. Characterization of mouse antisera was performed as described for the rabbit pAbs. Different serial dilutions of the sera were evaluated by single determinations due to the limited sample volume that can be extracted from the mouse at this stage. **Table 1** shows the results for the different antisera collected after the third injection and diluted to afford an A_{max} value close to 1.0. The titers for these antisera enter into a normal-to-high range for this animal source. On the contrary, IC_{50} values in the low nanomolar range (even below 10 nM CPPU in one case) were found, which are unusually low for mouse antisera. Antisera from mice immunized with BSA−p2 conjugate (Mp2) gave more sensitive assays than those from mice immunized with BSA−p6 conjugate (**Table 1**). Only antiserum Mp6#3 rendered assays comparable to those obtained with the Mp2-type antisera. The assay sensitivity achieved with antisera of the Mp6 type was in the low nanomolar range only if a conjugate containing a short linker (p2) was employed at 0.1 μg/mL as coating conjugate. The linker length did not seem to be so important with antisera of the Mp2 type. Finally, mouse antisera collected after the fourth injection were also evaluated. Unlike sera collected after the third injection, the blood was extracted 4 days after the fourth injection, which corresponds to the peak of lymphocyte proliferation and not to the peak of antibody production. For this reason, the titers were lower than those observed after the previous injection. However, the IC_{50} values remained comparable; below 10 nM for most cases when the coating conjugate was used at 0.1 μg/mL (results not shown).

Mouse Monoclonal Antibodies. Regarding the four fusions performed from mice immunized with p2, the growth yields were 88, 63, 73, and 89%, respectively. A collection of 15 p2-derived mAbs was thereafter purified. On the other hand, growth yields of the four fusions from mice immunized with the BSA−p6 conjugate were between 60 and 80%, and a collection of 10 p6-derived mAbs was finally produced. All of the

Table 2. Evaluation of Rabbit Antisera with the Two-Step ic-ELISA

		rabbit antisera[a]					
		Rp6#1			Rp6#2		
OVA conjugate	μg/mL	dil[b]	A_{max}	IC_{50}[c]	dil	A_{max}	IC_{50}
p2	0.1	2000	0.83	1.4	2000	0.68	2.9
		1000	1.59	1.2	1000	1.16	4.8
	1.0	2000	0.68	47.5	1000	0.91	101.2
		1000	1.22	46.7	300	2.71	179.6
p6	0.1	1000	0.86	0.6	1000	0.70	0.9
		300	2.75	0.6	300	2.05	1.0
	1.0	2000	0.72	5.0	2000	0.67	10.0
		1000	1.40	5.1	1000	1.25	10.1
CdPUp6	0.1	300	0.44	0.3	100	0.77	0.4
		100	1.05	0.4	30	1.85	0.8
	1.0	1000	0.53	0.6	1000	0.33	0.7
		300	1.90	0.6	300	1.08	0.7
PPUp6	1.0	30	0.76	1.1	30	0.82	0.3
		10	1.54	2.7	10	1.41	1.4

[a] Average assay parameters of three independent experiments. [b] Dilution factors of sera ($\times 10^3$). [c] Average values in nM for CPPU as competitor.

produced mAbs were of the IgG_1 isotype containing κ-type light chains. Therefore, two batteries of mAbs against CPPU have been generated; one from a conjugate with a rather short spacer arm and one from the equivalent conjugate but with a medium-sized linker.

Two-Step Conjugate-Coated ic-ELISAs. Bidimensional competitive assays for CPPU were performed with rabbit pAbs and mouse mAbs using homologous and heterologous conjugates in the indirect ELISA format with a two-step procedure.

Two-Step Indirect Assays Using pAbs. Rabbit antisera at different dilutions were evaluated using this format with the homologous and three heterologous coating conjugates at two different concentrations. The results for the two antisera dilutions that afforded the A_{max} values immediately above and below 1.0 are listed in **Table 2**. In general, both antisera showed very high titers and high sensitivity for CPPU, with IC_{50} values below 1.0 nM calculated from standard curves with slopes below −1.0. Antiserum Rp6#1 afforded more sensitive assays than antiserum Rp6#2 with all coating conjugates, with the only exception of OVA−PPUp6. The linker length heterology did not afford an improvement of the IC_{50} values, as also observed by other authors (*26*). Interestingly, for most coating conditions, the two dilutions of the same antiserum gave equivalent IC_{50} values, despite the important differences observed in the A_{max} values of some curves. Likewise, assay sensitivities greatly improved upon reduction of the concentration of conjugates OVA−p2 and OVA−p6, but not with conjugates introducing structural heterologies (OVA−CdPUp6 and OVA−PPUp6). If the two conjugates with structural heterologies are compared, the withdrawal of the chlorine atom (PPUp6) reduced considerably the titer of the assay. In fact, no assay could be executed at 0.1 μg/mL of OVA−PPUp6. Altogether, several combinations of conjugates and pAbs could be used to develop immunoassays with sensitivities for CPPU in the subnanomolar range while keeping the background signal below 0.1 absorbance unit. As an example, the normalized competitive curve obtained with pAb Rp6#1 [(5×10^5)-fold dilution in PBST] and OVA−p6 (0.1 μg/mL) is shown in **Figure 3A** (squares), which presents a slope of −0.7. This assay showed a LOD of 0.020 ± 0.003 nM.

Two-Step Indirect Assays Using mAbs. This study was performed with all of the mAbs; the results with four selected p2-derived antibodies are shown in **Table 3**, and those for the most sensitive p6-derived mAbs are listed in **Table 4**. Overall, no assay could be run with OVA−PPUp6, whereas with the other conjugates IC_{50} values below 1.0 nM were easily found. It could be observed that all mAbs showed a strong binding to the conjugate OVA−p2, independent of their origin. In fact,

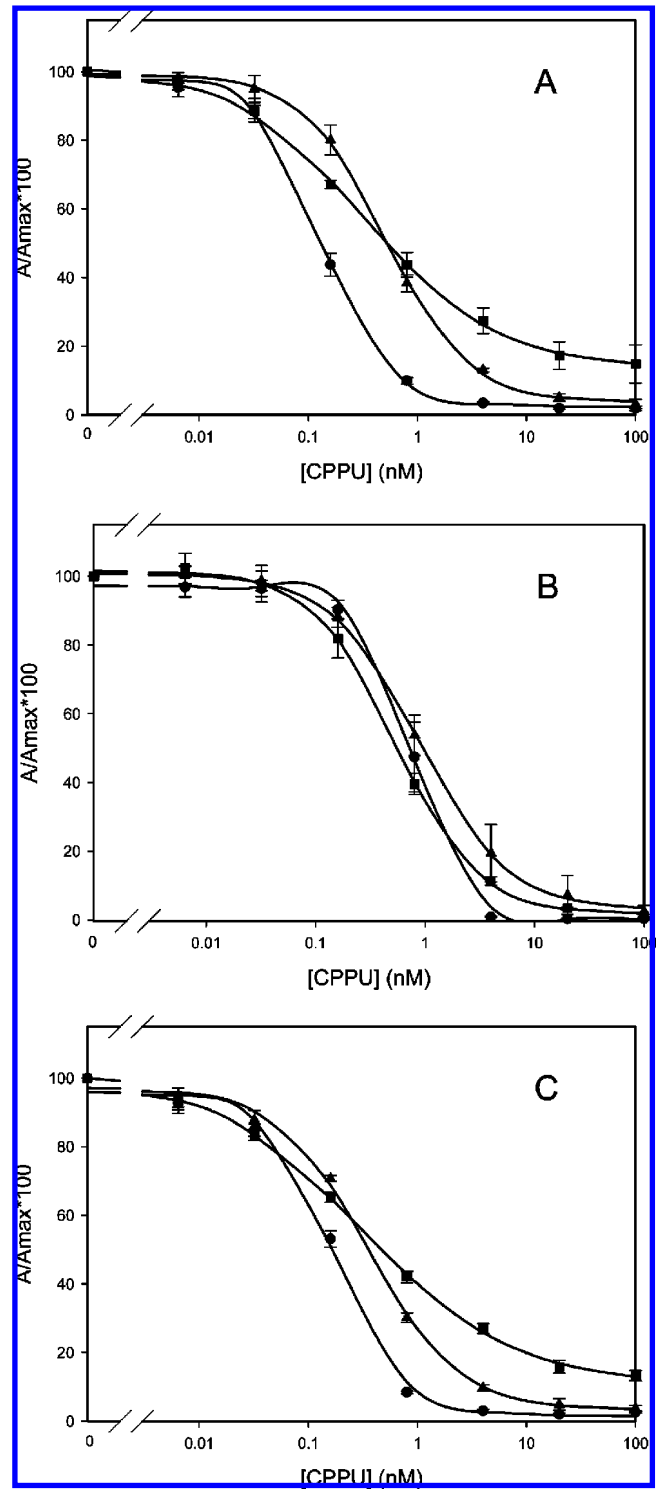

Figure 3. Standard curves for three antibodies (squares, pAb Rp6#1; triangles, mAb p2#51; circles, mAb p6#42) in three different assays. The curves are the mean of three independent determinations. (**a**) Assays in the two-step ic-ELISA. Mean A_{max} values were 0.9, 1.1, and 0.9 for Rp6#1, p6#42, and p2#51, respectively. (**b**) Assays in the one-step ic-ELISA. Mean A_{max} values were 1.6, 1.1, and 0.9 for Rp6#1, p6#42, and p2#51, respectively. (**c**) Assays in the dc-ELISA format. Mean A_{max} values were 1.2 for all antibodies.

Table 3. Assay Parameters of Four mAbs, Obtained from Mice Immunized with BSA−p2, Using Different Coating Conjugates and CPPU as Competitor in the Two-Step ic-ELISA

		monoclonal antibody[a]											
		p2#21			p2#51			p2#52			p2#66		
OVA conjugate	μg/mL	concn[b]	A_{max}	IC_{50}[c]	concn	A_{max}	IC_{50}	concn	A_{max}	IC_{50}	concn	A_{max}	IC_{50}
p2	0.1	10	0.59	0.7	10	0.52	0.5	10	0.95	1.8	10	0.46	0.5
		30	1.41[d]	0.9[d]	30	1.39	0.6	30	2.15	2.5	30	1.13	0.7
	1.0	10	0.62	7.9	10	0.74	2.6	10	ni[e]	ni	30	0.94	8.3
		30	1.43	9.0	30	1.67	2.9	30	ni	nl	100	2.35	8.7
p6	0.1	100	0.75	0.7	100	0.80	0.7	10	0.63	0.4	100	0.91	0.5
		300	1.22	1.5	300	1.36	1.8	30	1.45	0.6	300	1.45	1.2
	1.0	10	0.59	0.7	10	0.57	0.6	10	0.80	3.7	10	0.38	1.4
		30	1.24	0.8	30	1.38	0.7	30	1.98	3.7	30	1.04	1.0
CdPUp6	1.0	300	nr[f]	nr	30	0.71	0.4	100	0.77[d]	0.4[d]	300	nr	nr
		nd	nd	nd	100	1.62	0.8	300	1.33[d]	1.2[d]	nd	nd	nd
PPUp6	1.0	300	nr	nr	300	nr	nr	300	nr	nr	300	nr	nr

[a] Average assay parameters of three independent experiments. [b] Antibody concentration in ng/mL. [c] Mean values in nM. [d] Average of two replicates. [e] No inhibition. [f] Not recognized.

Table 4. Assay Parameters of Four mAbs, Obtained from Mice Immunized with BSA–p6, Using Different Coating Conjugates and CPPU as Competitor in the Two-Step ic-ELISA

		monoclonal antibody[a]											
		p6#24			p6#32			p6#41			p6#42		
OVA conjugate	μg/mL	concn[b]	A_{max}	IC_{50}[c]	concn	A_{max}	IC_{50}	concn	A_{max}	IC_{50}	concn	A_{max}	IC_{50}
p2	0.1	10	0.46	0.2	30	0.80	0.8	10	0.41	0.2	30	0.98	0.1
		30	1.16	0.4	100	1.97	1.1	30	1.14	0.3	100	2.59	0.3
	1.0	10	0.42	4.7	30	0.60	10.4	10	0.68	1.6	30	0.73	0.6
		30	1.09	4.6	100	1.62	11.3	30	1.68	2.0	100	2.22	0.8
p6	0.1	30	0.71[d]	0.2[d]	100	0.81	0.4	100	0.97	0.8	30	0.38	0.1
		100	1.46[d]	0.6[d]	300	1.38	1.2	300	1.53	2.3	100	1.02	0.3
	1.0	10	0.40	0.7	30	0.76[e]	1.2[e]	10	0.49	0.4	30	0.86	0.1
		30	1.04	1.0	100	2.00	1.6	30	1.34	0.5	100	2.45	0.4
CdPUp6	1.0	30	0.48	0.1	300	nr[f]	nr	30	0.30	0.6	30	0.28	0.1
		100	1.28	0.4	nd	nd	nd	100	1.35	0.6	100	1.08	0.2
PPUp6	1.0	300	nr	nr	300	nr	nr	300	nr	nr	300	nr	nr

[a] Average assay parameters of four independent experiments. [b] Antibody concentration in ng/mL. [c] Mean value in nM. [d] Average of two determinations. [e] Average of three determinations. [f] Not recognized.

equivalent antibody concentrations were required to obtain the same signal at 0.1 and 1.0 μg/mL of this conjugate. As it had been already observed with rabbit pAbs, the chloropyridyl ring plays a fundamental role in conjugate recognition by the mAbs. The change of the pyridyl ring to a phenyl ring appears to be an important heterology, and three of the mAbs did not recognize the conjugate OVA−CdPUp6, not even at 1.0 μg/mL. Nevertheless, those mAbs that bound this conjugate afforded assays with IC_{50} values comparable to those achieved with the homologous conjugate, but with higher slopes. No assay could be carried out with the conjugate OVA−CdPUp6 at 0.1 μg/mL with any of the selected mAbs. Therefore, heterologies did not mean an improvement in the final sensitivity of these assays.

As usually observed, the fitted curves presented higher slopes with mAbs than with pAbs (they were mainly over −1.0 and typically around −1.3). The steepest slopes were commonly obtained with the homologous conjugates at low coating concentration (OVA−p2 and OVA−p6 at 0.1 μg/mL) or with the heterologous conjugate (OVA−CdPUp6) at 1.0 μg/mL. In all of these experiments, the background signal was kept low, typically below 0.1 absorbance unit. The lowest IC_{50} value was attained with 30 ng/mL of mAb p6#42 and the homologous coating conjugate at 1.0 μg/mL. The normalized inhibition curve obtained under those conditions is depicted in **Figure 3A** (circles), which had a slope of −1.4. The calculated LOD value for CPPU in this assay was 0.030 ± 0.006 nM. Regarding the p2-derived mAbs, the normalized standard curve obtained with 30 ng/mL of p2#51 and 0.1 μg/mL of OVA−p2 is shown in **Figure 3A** (triangles), which had a slope of −1.1. This assay presented a LOD of 0.079 ± 0.025 nM for CPPU.

Cross-Reactivity and Binding Studies. The binding of polyclonal and monoclonal antibodies to a series of compounds with cytokinin-like activity was studied. No cross-reactivity was observed with dPU, KIN, ZEA, and 6-BAP, so no interferences from these compounds potentially present in real samples are expected if these antibodies are used for the analysis of CPPU in food. Concerning TDZ, a herbicide with cytokinin activity and widely used as defoliant in cotton crops (**Figure 1**), certain cross-reactivity (10−25%) was observed with some mAbs, which would allow using these particular antibodies for the determination of this compound.

CPPU is a molecule consisting of two aromatic rings linked by a urea bridge, with the pyridyl ring bearing a chlorine atom. This certain symmetry makes CPPU a suitable compound to study the importance of the different moieties of the analyte in the antibody binding event. With this aim, commercial and intentionally synthesized compounds, structurally analogous to CPPU, were assayed in the ic-ELISA using the homologous coating conjugate. **Figure 4** displays the CR pattern for one rabbit antiserum and for three mAbs selected as representative of the different observed behaviors. The two rabbit antisera generated in this study, Rp6#1 and Rp6#2, showed equivalent CR patterns. Also, most mAbs essentially shared the CR pattern

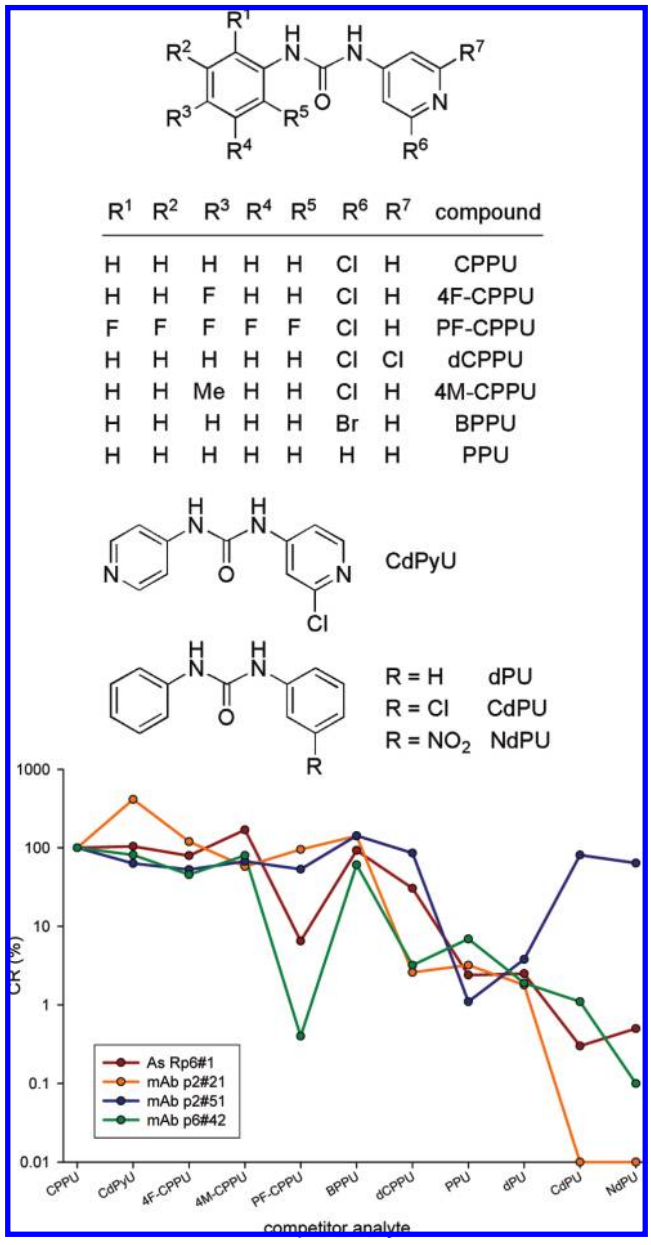

Figure 4. Chemical structures of compounds analogous to CPPU and their CR patterns for one pAb and three representative mAbs.

shown by the pAbs and mAb p6#42. For this group of antibodies, modifications of the phenyl ring, such as the introduction of a fluorine atom (4F-CPPU) or a methyl group (4M-CPPU) at position R^3 (where the immunizing hapten bears the spacer arm), or even its substitution by a pyridyl ring (CdPyU), had little influence on antibody binding. Nevertheless, more severe modifications of the phenyl group, such as the introduction of five fluorine atoms, had varied effects on mAbs, whereas the pAbs showed recognition to an intermediate level. Therefore, it can be concluded that moderate modifications of the CPPU structure at the ring used for hapten functionalization (proximal site) are well tolerated by most antibodies. On the contrary, modifications at the distal site had a relevant influence on antibody binding. In this respect, the elimination of the chlorine atom at the pyridyl ring (PPU) or the change of the chloropyridyl ring by a chlorophenyl ring (CdPU) severely disturbed the interaction, resulting in poor recognition of the corresponding competitors (CR under 10%). Therefore, not surprisingly, substitution of the chloropyridyl group by a nitrophenyl ring (NdPU) or its replacement by a phenyl group

Table 5. Bidimensional Competitive Study in the One-Step ic-ELISA

immunoreagent	conj[a]	Ab[b]	A_{max}[c]	IC_{50}[d]
Rp6#1 + OVA–p2	0.1	1000	0.44	0.5
		300	1.57	0.4
p2#51 + OVA–p2	0.1	30	0.71	0.3
		100	1.63	0.6
p6#42 + OVA–p6	1.0	30	0.53	0.2
		100	1.66	0.4

[a] Concentration of coating conjugate in μg/mL. [b] Antiserum dilution ($\times 10^3$) or mAb concentration in ng/mL. [c] Average results of three independent experiments. [d] Values in nM for CPPU as competitor.

(dPU) also had a strong impact on antibody binding (CR around 2%). It is worth mentioning that all mAbs were unable to differentiate between CPPU and its brominated analogue (BPPU), which proves the importance of the presence of a halogen atom at this position of the pyridyl ring, whereas the presence of two chlorine atoms (dCPPU) had a diverse effect depending on the antibody under consideration, with the pAbs showing an intermediate behavior.

Antisera, as far as they are a heterogeneous mixture of raw immunoglobulins, contain all of the binding possibilities selected in vivo by the immune system, so they constitute an appropriate model to elucidate the relationship between the structure of the immunizing hapten and the selectivity of the derived antibodies. On the contrary, monoclonals represent a single picture of the whole immunological response in the mouse, so mAbs with quite distinct paratopes may be formed and eventually selected. Thus, whereas most mAbs displayed a selectivity pattern in line with that of the pAbs, mAbs p2#21 and p2#51 behaved differently, mainly concerning recognition of the distal part of the CPPU molecule (the chloropyridyl ring). The selectivity of mAb p2#21 was strictly in accordance with Landsteiner's principle; that is, modifications of the analyte structure at the farthest position from the functional group used to link it to the carrier protein drastically affect antibody recognition (*27*). This fact is particularly evident with competitors CdPU and NdPU. On the contrary, mAb p2#51 seems to follow this principle in a less strict way, according to the high affinity showed by this antibody to CdPU and NdPU (CR around 100%) and the low binding to PPU (CR around 1%). It seems that binding to this antibody requires the presence of a substituent group (chlorine, bromine, or nitro) at the distal ring, either phenyl or pyridyl.

One-Step Conjugate-Coated ic-ELISAs. Assays were also developed using the ic-ELISA format with only one incubation step. The low concentration of specific immunoreagents usually demanded by the ic-ELISA format, the possibility to preincubate the sample with the antibody, and the higher stability of the immunoreagents that are used make this format preferable to the antibody-coated dc-ELISA format. Nevertheless, the additional incubation step with a secondary antibody has usually been considered to be a drawback. The good performance of the one-step ic-ELISA has been demonstrated before (*28, 29*). As a novelty, we have applied this assay format, with only a 1 h incubation step, not only to mouse mAbs but also to rabbit pAbs. Competitive assays with mAbs were done with RAM−HRP conjugate at 1/2000 final dilution, whereas for pAbs a 1/10000 final dilution of GAR−HRP was employed. Mixtures were prepared as described under Materials and Methods. For the assays with pAbs, the antiserum and the secondary antibody mixture required, before addition to the plate, at least a 10 min incubation time at room temperature to stabilize the signal. **Table 5** lists the curve parameters for one pAb, one p2-derived mAb, and one p6-derived mAb under optimized assay conditions. Interestingly, sensitive competitive assays could be

Table 6. Assay Parameters in the dc-ELISA Format

antibody		enzyme tracer[a]								
		p2–HRP			p6–HRP			CdPUp6–HRP		
		concn[b]	A_{max}	IC_{50}[c]	concn	A_{max}	IC_{50}	concn	A_{max}	IC_{50}
pAbs	Rp6#1	10	0.89	0.4	3	0.91	2.7	10	0.66	0.7
		30	1.68	0.7	10	2.78	2.1	30	1.34	1.0
mAbs	p2#51	300	nr[d]	nr	3	0.86[e]	0.7[e]	3	0.49[e]	0.9[e]
		nd	nd	nd	10	2.38	0.6	10	1.22	1.1
	p2#66	10	0.96	0.2	nd	nd	nd	300	nr	nr
		30	1.20	0.4	3	1.42[e]	0.9[e]	nd	nd	nd
	p6#24	300	nr	nr	3	0.88	0.7	10	0.52	0.4
		nd	nd	nd	10	2.52	0.7	30	1.27	0.4
	p6#42	3	0.48	0.5	nd	nd	nd	10	0.95	0.5
		10	1.39	0.4	3	1.02	0.9	30	2.43	0.5

[a] Average results of three independent experiments. [b] Concentration of the enzyme tracer in ng/mL. [c] Mean value in nM for CPPU as competitor. [d] Not enough signal. [e] Average of two replicates.

developed using rabbit pAbs in this format. The IC_{50} values obtained with these antibodies for CPPU were in all cases below 0.5 nM, which are very similar to the values found with the same antibodies and the same assay conjugates in the two-step ic-ELISA. Even the concentrations of the immunoreagents were equivalent for the two-step and one-step assays (**Tables 2**−**5**). The normalized inhibition curves for three selected assays are shown in **Figure 3B**. The assay with the antiserum Rp6#1 [(5×10^5)-fold dilution in PBST] was run with OVA−p2 coating conjugate at 0.1 μg/mL. For the assay with mAb p6#42 (50 ng/mL), plates were coated with 1.0 μg/mL of OVA−p6 conjugate. Finally, 0.1 μg/mL of OVA−p2 conjugate was employed when assays with mAb p2#51 (40 ng/mL) were run. Regarding the slopes of the inhibition curves, no differences were found between these two procedures of the ic-ELISA format. The calculated LODs for CPPU using the three chosen antibodies were 0.013 ± 0.001, 0.036 ± 0.005, and 0.048 ± 0.002 nM for Rp6#1, p6#42, and p2#51, respectively.

Antibody-Coated dc-ELISAs. Haptens p2, p6, and the heterologous CdPUp6 and PPUp6 were coupled to HRP and included in this study. The optimum concentration of each tracer conjugate was determined for a fixed coating concentration of each monoclonal or polyclonal antibody. A bidimensional assay was performed with different dilutions of coating pAb and tracer conjugate. For Rp6#1, 1/20000 was found as the optimum dilution. The results of representative competitive assays for five antibodies are shown in **Table 6**. It is known that short spacer arms may not render a measurable signal in the dc-ELISA (*30, 31*). Interestingly, the two pAbs and some mAbs recognized the tracer with short spacer (HRP–p2) and also the heterologous conjugate HRP−CdPUp6. No assay could be obtained with any of the antibodies using the HRP−PPUp6 tracer. The normalized inhibition curves for a pAb and two mAbs are shown in **Figure 3C**. The obtained IC_{50} values for CPPU under the selected assay conditions were below 1.0 nM, slightly higher than those of the ic-ELISAs. It is worth mentioning that the assay with pAb Rp6#1 and HRP−p2 (10 ng/mL) afforded an inhibition curve with a slope around −1.1, steeper than the slope of the curves obtained with this pAb in the indirect assays. The dc-ELISA with this antiserum under these conditions showed a LOD for CPPU of 0.082 ± 0.023 nM. The curve with the steepest slope (−2.0) was obtained with mAb p6#42 and tracer HRP−CdPUp6 (5 ng/mL)—as also observed with the two previous assays using this antibody—and, consequently, this assay rendered the highest LOD (0.258 ± 0.066 nM). Finally, the assay with mAb p2#51 and 5 ng/mL of HRP−p6 rendered a competitive curve with a slope of −1.1 and a LOD for CPPU of 0.121 ± 0.028 nM.

A synthetic strategy has been designed to link linear aliphatic spacers to aromatic rings of the target molecule. In addition, a collection of high-affinity mAbs and two pAbs for CPPU have been obtained, which may be useful for the monitoring of this synthetic phytohormone. A thorough investigation has been performed with a large variety of newly synthesized chemical compounds with structural analogies to CPPU. Antisera and most monoclonal antibodies bound compounds with modifications at the proximal ring, whereas changes at the distal ring meant a decreased binding. Nevertheless, exceptions to this behavior were found and some mAbs accepted different substituents at a distal position. Interestingly, although most antibodies recognized similarly CdPU and PPU analogues in solution, sensitive assays with sufficient maximum signals were just obtained in both ELISA formats with conjugates of the CdPU hapten (CdPUp6). In this respect, it was clear that rabbit pAbs tolerated conjugates with structural heterologies better than mouse monoclonals. It could also be concluded that no difference was observed between the mAbs obtained from mice immunized with conjugates containing two-carbon or six-carbon spacers. A set of immunoassays has been characterized with different mAbs and pAbs in the indirect and direct formats. The ic-ELISAs have been developed following two-step and one-step procedures, affording equivalent LODs with very similar immunoreagent requirements even for the pAbs. An important variation in the slope of the inhibition curves was found between the ic-ELISA and the dc-ELISA for the pAbs. Outstandingly, most of the assays developed in this work attained detection limits in the low picograms per milliliter range.

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Supporting Information Available: Synthesis of haptens and competitors, conjugate preparation, and antibody production. This material is available free of charge via the Internet at http://pubs.acs.org.

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