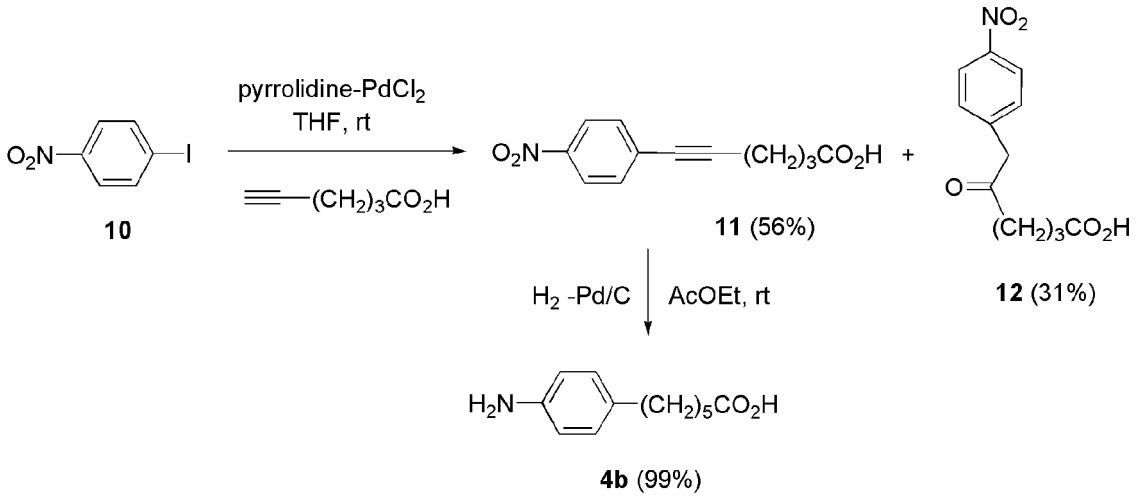
Supporting Information

Production and Characterization of Monoclonal and Polyclonal Antibodies to Forchlorfenuron

C. Suárez-Pantaleón, J.V. Mercader, C. Agulló, A. Abad-Somovilla, and A. Abad-Fuentes

1. Synthesis of Haptens and Competitors

Preparation of 6-(4-aminophenyl)hexanoic Acid (4b).



6-(4-Nitrophenyl)hex-5-ynoic Acid (11). To a solution of 1-iodo-4-nitrobenzene (**10**) (966 mg, 3.88 mmol) in anhydrous THF (5 mL) was added pyrrolidine (1.7 mL) and $PdCl_2$ (7.9 mg, 0.045 mmol) and the mixture was stirred for a few minutes. Then, hex-5-ynoic acid (400 μL, 396 mg, 3.53 mmol) was added and the mixture was stirred at rt for 24 h, then poured into water and extracted one with EtOAc. The aqueous phase was cooled in an ice bath and carefully acidified with 1M hydrochloric acid to pH 2-3 and then extracted with EtOAc. The combined organic layers were washed with water and brine and dried over $MgSO_4$ and concentrated. Column chromatography, using chloroform as eluent, afforded the acetylenic acid **11** (464 mg, 56%) as an orange solid.

Mp 87-88 ºC (from ether-hexane). ^{1}H NMR ($CDCl_3$) δ: 8.16 (2H, dd, J = 8.7 Hz, H-3' and H-5'), 7.52 (2H, dd, J = 8.7 Hz, H-2' and H-6'), 2.56 (4H, two overlapped t, *J* = 7.3 Hz, H-2 and H-4), 1.97 (2H, q, *J* = 7.3 Hz, H-3). ^{13}C NMR ($CDCl_3$) δ: 178.47 (C-1), 146.73 (C-4'), 132.30 (C-1'), (C-2'/C-6'), 130.63 (C-4'), 123.51 (C-3'/C-5'), 94.60 (C-5), 80.20 (C-6), 32.60 (C-2), 23.21 (C-3), 18.89 (C-4). IR (KBr): 3200-2500, 2225, 1710, 1691, 1593, 1535, 1351, 856, 747 cm^{-1}. MS (EI) *m/z*: 233 (M^+, 27), 216 (27), 205 (14), 192 (11), 191 (100), 186 (37), 174 (59), 160 (11). HRMS: calcd for $C_{12}H_{11}NO_4$ 233.06881, found 233.06789.

Further elution with the same eluent afforded 6-(4-nitrophenyl)-5-oxohexanoic acid (**12**) (274 mg, 31%), also as a solid. Mp 107-109 ºC (from ether-hexane). ^{1}H NMR ($CDCl_3$) δ: 10.1 (1H, OH), 8.19 (2H, dd, *J* = 8.7 Hz, H-3' and H-5'), 7.36 (2H, dd, *J* = 8.7 Hz, H-2' and H-6'), 3.83 (2H, s, H-6), 2.62 (2H, t, *J* = 7.2 Hz, H-4), 2.39 (2H, t, *J* = 7.2 Hz, H-2), 1.92 (2H, q, *J* = 7.2 Hz, H-3). ^{13}C NMR (CDCl3) δ: 205.56 (C-5), 177.84 (C-1), 147.08 (C-4'), 141.40 (C-1'), 130.59 (C-2' and C-6'), 123.98 (C-3' and C-5'), 49.45 (C-6), 41.35 (C-4), 32.52 (C-2), 18.51 (C-3). IR (KBr): 3200-2600, 1716, 1697, 1518, 1345, 1278, 1094, 861, 703 cm^{-1}. MS (EI) *m/z*: 234 (3%), 233 (4), 137 (100), 136.10 (115.53), 107 (12), 90 (17). HRMS (EI): calcd for $C_{12}H_{12}NO_4$ [$(M^+$+1)-H_2O] 234.07663, found 234.07601.

6-(4-Aminophenyl)hexanoic Acid (**4b**)**.** A solution of the above obtained acetylenic acid **11** (375 mg 1.608 mmol) in AcOEt (11 mL) and 10% Pd/C (92 mg) was hydrogenated at rt under a hydrogen pressure of 64 psi for 3-4 hours. The reaction mixture was filtered through a short Celite pad and the filter cake was washed with EtOAc. The filtrate and the washings were combined and concentrated under vacuum to afford nearly pure 6-(4-aminophenyl)hexanoic acid (**4b**) as a white solid (328 mg, 99%), which was used without further purification. Mp 107-108 ºC (from $CHCl_3$) [Lit.[1] 107-108 ºC, Lit.[2] 108-109 ºC]. ^{1}H NMR ($CDCl_3$) δ: 6.96 (1H, d, *J* = 8.3 Hz, H-2' and H-6'), 6.63 (1H, d, *J* = 8.3 Hz,H-3' and H-5'), 5.72 (3H, br s, OH and NH_2), 2.51 (2H, t, *J* = 7.4 Hz, H-6), 2.34 (2H, t, *J* = 7.4 Hz, H-2), 1.72-1.53 (4H, m, H-3 and H-5), 1.36 (2H, m, H-4). ^{13}C NMR ($CDCl_3$) δ: 179.71 (C-1), 144.00 (C-4'), 132.93 (C-1'), 129.27

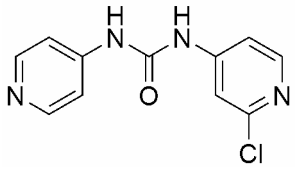
---

(1) Kornblum, N.; Iffland, D. C. Selective replacement of the aromatic primary amino group by hydrogen in aromatic-aliphatic diamines. *J. Am. Chem. Soc*.**1949**, *71*, 2137-2143.

(2) van der Scheer, J. Preparation of γ-(p-aminophenyl)butyric acid and ε-(p-aminophenyl)caproic acids. *J. Am. Chem. Soc*. **1934**, *56*, 744-745.

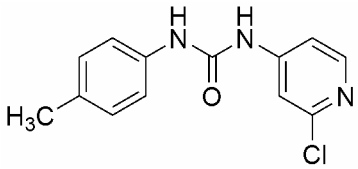
(C-2' and C-6'), 115.52 (C-3' and C-5'), 34.92 and 34.09 (C-2 and C-6), 31.49 (C-5), 28.75 (C-4), 24.68 (C-3). IR (KBr): 3336, 3177, 3037, 2931, 2851, 2700-2400, 1697, 1615, 1516, 1217, 956, 487 cm^{-1}. MS (EI) *m/z* (%): 207 (M^+, 100), 148 (2), 132 (5), 119 (11), 107 (48). HRMS: calcd for $C_{12}H_{17}NO_2$ 207.12593, found 207.12592.

Analogous Compounds



(CdPyU)

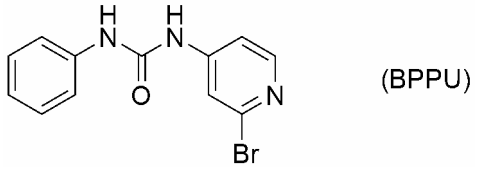
1-(2-Chloropyridin-4-yl)-3-(pyridine-4-yl)urea (CdPyU). 2-Chloropyridin-4-amine (49.6 mg, 0.39 mmol) was added as a solid to a solution of acyl azide **9** (63.0 mg, 0.43 mmol) in anhydrous benzene (5.5 mL) under argon. The reaction mixture was stirred for 3.5 h at reflux and then cooled to 15 ºC. The solid formed was collected by filtration and purified by column chromatography, using CH_3Cl:MeOH 9:1 as eluent, to give pure CdPyU (49.3 mg, 52% yield) as a as a slightly orange solid. Mp 185-190 ºC (from acetone). ^1H NMR (acetone-d_6) 8.94 and 8.80 (1H each, each br s, two NH), 8.41 (2H, br d, J = 6.2 Hz, H-2/H-6 Py), 8.19 (1H, d, J = 5.6 Hz, H-6 ClPy), 7.73 (1H, d, J = 1.8 Hz, H-3 ClPy), 7.52 (2H, br d, J = 6.2 Hz, H-3/H-5 Py), 7.40 (1H, dd, J = 5.6, 1.8 Hz, H-5 ClPy); ^{13}C NMR (acetone-d_6), δ 153.62 (NCON), 152.25 (C-2/C-6 Py overlapped with C-4 ClPy), 151.86 (C-6 ClPy), 150.54 (C-4 Py), 147.77 (C-2 ClPy), 114.56, 113.82, 113.89 and 113.77 (C-3 ClPy, C-5 ClPy, C-3/C-5 Py); IR (KBr) 3350, 3279, 1733, 1574, 1509, 1383, 1268, 1176, 990, 853 and 722 cm^{-1}; MS (EI) *m/z* (%): 248 (3), 207 (2), 205 (3), 156 (32), 154 (100), 131 (2), 130 (21), 129 (7), 128 (67), 121 (10), 120 (85), 119 (56); HRMS: calcd for $C_{11}H_9ClN_4O$ 248.04649, found 248.04651.



(4M-CPPU)

1-(2-Chloropyridin-4-yl)-3-p-tolylurea (4M-CPPU). *p*-Tolyl isocyanate (108.0 μL, 0.86 mmol) was added via syringe to a solution of 2-chloropyridin-4-amine (100.0 mg, 0.78 mmol) in dry benzene (1.0 mL) and the mixture was stirred at reflux for 5 h and then cooled to 15 ºC. The solid obtained after filtration was purified by silica gel

chromatography, using CH_3Cl as eluent, to give pure 4M-CPPU (116.8 mg, 57% yield) as a yellow solid. Mp 188-192 ºC (from MeOH) [Lit.[3] 185.5-198 ºC]. ^{1}H NMR (DMSO-d_6) δ 9.31 and 8.87 (1H each, each s, two NH), 8.17 (1H, d, *J* = 5.6 Hz, H-6 Py), 7.64 (1H, d, *J* = 1.8 Hz, H-3 Py), 7.34 (2H, d, *J* = 8.3 Hz, H-2/H-6 Ph), 7.29 (1H, dd, *J* = 5.6, 1.8 Hz, H-5 Py), 7.11 (2H, d, *J* = 8.3 Hz, H-3/H-5 Ph), 3.37 (CH_3); ^{13}C NMR (DMSO-d_6), δ 151.79 and 150.85 (NCON and C-4 Py), 149.87 (C-6 Py), 149.15 (C-2 Py), 136.11 (C-1 Ph), 131.54 (C-4 Ph), 129.17 (C-3/C-5 Ph), 118.77 (C-2/C-6 Ph), 111.76 and 111.08 (C-3 and C-5 Py), 20.29 8 (CH_3); IR (KBr) 3376, 3230, 3027, 2924, 1729, 1592, 1572, 1527, 1493, 1382, 1248, 1184, 1039, 991, 849 and 803 cm^{-1}; MS (EI) *m/z* (%):261 (20), 243 (3), 156 (10), 155 (2), 154 (29), 134 (9), 133 (100), 132 (47), 131 (1), 129 (4), 128 (63), 119 (13); HRMS: calcd for $C_{13}H_{12}ClN_3O$ 261.06689, found 261.06793.



(BPPU)

1-(2-Bromopyridin-4-yl)-3-phenylurea (BPPU). Phenyl isocyanate (137.0 μL, 1.26 mmol) was added dropwise to a solution of 2-bromopyridin-4-amine (197.5 mg, 1.14 mmol) in anhydrous CH_2Cl_2 (2.0 mL) and the mixture was stirred at room temperature for 15 h. The reaction mixture was then diluted with hexane and the precipitated solid formed was separated by filtration and purified by silica gel chromatography, using CH_3Cl as eluent, affording pure BPPU (199.6 mg, 60% yield) as a brownish solid. Mp 187-189 (from MeOH-H_2O) [Lit.[4] 186-189 ºC]. ^{1}H NMR (DMSO-d_6) δ 9.40 and 8.98 (1H each, each s, two NH), 8.15 (1H, d, *J* = 5.7 Hz, H-6 Py), 7.81 (1H, d, *J* = 1.8 Hz, H-3 Py), 7.46 (2H, dd, *J* = 8.5, 1.1 Hz, H-2/H-6 Ph), 7.34 (1H, dd, *J* = 5.7, 1.8 Hz, H-5 Py), 7.30 (2H, br t, *J* = 7.5 Hz, H-3/H-5 Ph), 7.02 (1H, tt, *J* = 7.5, 1,1 Hz, H-4 Ph); ^{13}C NMR (DMSO-d_6), δ 151.76 (NCON), 150.34 (C-6 Py), 148.74, 141.92 and 138.70 (C-2 Py, C-4 Py and C-1 Ph), 128.79 (C-3/C-5 Ph), 122.62

---

(3) Okamoto, T.; Isogai, Y.; Shudo, K.i; Takahashi, S. *N*-(2-Chloro-4-pyridyl)ureas useful in plant growth regulating compositions. DE Patent Nº 2843722 A1, 1979.

(4) Okamoto, T.; Shudo, K.; Isogai, Y.; Takahashi, S. 4-Pyridyl ureas or thioureas substituted in position 2 and plant growth regulants containing them. EP patent Nº 10770 A1, 1980.

(C-4 Ph), 118.69 (C-2/C-6 Ph), 114.86 and 112.14(C-3 and C-5 Py); IR (KBr) 3395, 3240, 3126, 3037, 2962, 1730, 1580, 1529, 1495, 1192, 833 and 689 cm^{-1}.

2. Conjugate Preparation

Immunizing Conjugate. The active ester method was used to couple haptens p2 and p6 to BSA. Typically, a 100 mM p2 or p6 solution was prepared in DMF and mixed with 1 molar equivalent of NHS and 1 molar equivalent of DCC also in DMF. Additional DMF was added to bring the final concentration of all reagents to 50 mM. The hapten was activated overnight at room temperature in amber vials. The day after, the reaction was centrifuged and the supernatant was collected. Next, 300 µL of activated p2 hapten solution or 400 µL of activated p6 hapten solution was added dropwise to 2 mL of a 15 mg/mL BSA solution in conjugation buffer. The coupling reaction was allowed during 4 h at room temperature with moderate stirring. The initial hapten-to-protein molar ratio (MR) in the mixture was approximately 30:1 for p2 and 40:1 for p6. Finally, the conjugate was separated from uncoupled hapten by gel filtration on Sephadex G-25, using PB as eluent. The degree of hapten-to-protein conjugation was measured spectrophotometrically. If conjugation occurred, the UV–vis spectrum of the conjugate was slightly different from that of the free protein. Therefore, the final hapten-to-protein MR was the average calculated from the absorbance values at 280 nm and 260 nm by assuming that the molar absorption of the hapten and the protein were the same for the free and the conjugated forms. The purified conjugate was diluted to 1 mg/mL with PB and stored at –20 ºC.

Coating Conjugates. The mixed anhydride method was used to prepare conjugates of each hapten to OVA. Briefly, a 100 mM hapten solution was prepared in DMF and an aliquot was mixed with 1 molar equivalent of ICF and 1 molar equivalent of TBA also in DMF. The same solvent was added to bring the final concentration of all reagents to 90 mM. Hapten PPUp6 could not easily be dissolved in DMF, therefore a 6:4 (v/v) mixture of DMF:DMSO was used as solvent. In this case, all reagents were brought to a final concentration of 28 mM. Haptens were activated during 1 h at room temperature. Next, 100 µL of activated hapten solution was added dropwise to 2 mL of a 15 mg/mL OVA solution in conjugation buffer. For PPUp6, 200 µL for activated hapten were employed over 2 mL of a 7.5 mg/mL OVA solution. The coupling reaction was allowed during 2.5 h at room temperature with moderate stirring. The initial hapten-to-protein MRs in the mixtures were, in all cases, between 13 and 18 molecules of hapten per

molecule of protein. Finally, conjugates were separated from uncoupled haptens by gel chromatography as described for the immunizing conjugate and final hapten-to-protein MRs were estimated as before. Conjugates were stored at –20 ºC in amber vials at 1 mg/mL conjugate concentration.

Tracer Conjugates. The mixed anhydride method was also used to prepare conjugates of each hapten to HRP. Briefly, a 100 mM hapten solution was prepared in DMF and mixed with 1 molar equivalent of ICF and 1 molar equivalent of TBA also in DMF. The same solvent was added to bring the final concentration of all reagents to 66.5 mM for p2 and p6, or to 90 mM and 28 mM for CdPUp6 and PPUp6, respectively (PPUp6 could not be solubilized in DMF so a mixture of DMF and DMSO was employed as described before). Haptens were activated during 1 h at room temperature. Next, 100 µL of a 1/10 (v/v) dilution in DMF of activated hapten solution (except for activated PPUp6, which was diluted 1/3) was added dropwise to a 1 mL solution of HRP at 2.2 mg/mL in conjugation buffer. The coupling reaction was allowed during 4 h at room temperature with moderate stirring. The initial hapten-to-protein MRs in the mixtures were between 13 and 18 molecules of hapten per molecule of protein. The conjugates were separated from uncoupled haptens by gel chromatography as described before, and the final hapten-to-protein MRs were calculated using, in this case, the absorbance values of the conjugate at 400 and 280 nm. The purified conjugate was stored at –20 ºC in amber vials. A working solution was prepared by bringing the conjugate to a concentration of 0.5 mg/mL with storage buffer, and it was kept at 4 ºC in amber vials.

3. Antibody Production.

Immunization. Eight BALB/c female mice (8–10 weeks old) were immunized with the BSA–p2 or BSA–p6 conjugate by intraperitoneal injections. Doses consisted of an emulsion of 100 µL of PB containing 100 µg of hapten conjugate, estimated as protein concentration, 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. The antiserum from each mouse was obtained by submandibular bleeding nine or ten days after the third injection. Sera were diluted 1/10 with storage buffer and kept at 4 ºC in amber vials. 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 protein 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 µg/mL gentamicin (referred to as s-DMEM) and containing 10% (v/v) 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 4ºC. Cell fusion procedures were carried following standard procedures. After cytolysis of red blood cells by osmotic chock, 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. Twenty-four 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 simultaneous indirect noncompetitive and competitive ELISA with 1.0 µg/mL homologous coating conjugate. The immunological reaction was allowed during 2 h and the second reaction with an enzyme-labeled conjugate was run during 1 h. All other assay conditions were as herein described for the two-step assay format. The signal in noncompetitive conditions was compared with the competitive one when 1.0 or 0.1 µM CPPU was used as competitor. The ratio of both absorbances was used as the criterion for selecting the best antibody-secreting clones. The selected hybridomas were cloned by limiting dilution in HT cloning medium containing 20% FBS and 1% HFCS. Stable antibody-producing clones were expanded and cryopreserved in liquid nitrogen.

Purification of Monoclonal Antibodies. Immunoglobulins were purified from late stationary phase culture supernatants by ammonium sulfate precipitation and protein G affinity chromatography following the manufacturer's instructions. The column eluate was brought to 1 mg/mL with elution buffer. A fraction of the purified mAb solution was diluted with 1 volume of storage buffer and kept at 4 ºC in amber vials for daily usage. The remaining mAb solution was stored at 4 ºC as ammonium sulfate precipitate.