Electronic Supplementary Material (ESI) for Analyst.

SUPPLEMENTARY INFORMATION

Combined heterologies for monoclonal antibody-based immunoanalysis of fluxapyroxad

Eric Ceballos-Alcantarilla, Daniel López-Puertollano, Consuelo Agulló, Antonio Abad-Fuentes, Antonio Abad-Somovilla, Josep V. Mercader

Contents **Page**

General experimental procedures, reagents, and instruments

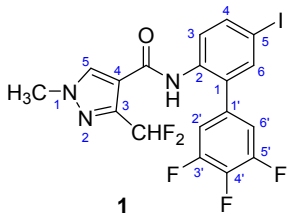
All operations involving air-sensitive reagents were performed under an inert atmosphere of dry nitrogen using syringe and cannula techniques, oven-dried glassware, and freshly distilled and dried solvents. The progress of reactions was monitored by thin layer chromatography (TLC) performed on F_{254} silica gel plates. The plates were visualized at 254 nm by immersion with aqueous ceric ammonium molybdate and heating. Column chromatography refers to flash chromatography and was performed on Merck silica gel 60, 230–400 mesh. All melting points were determined using a Kofler hot-stage apparatus and are uncorrected. IR spectra were recorded using a Nicolet Avatar 320 FT-IR spectrophotometer using liquid films or ATR for solids (IR band intensities: w = weak, m = medium, s = strong). ^{1}H NMR spectra were recorded on Bruker spectrometers, in the solvent indicated, at 300 MHz and ^{13}C NMR spectra at 75 MHz. ^{19}F NMR spectra were acquired at 282 MHz with high power proton decoupling. All proton and carbon spectra were referenced to residual solvent (^{1}H NMR: 7.26 ppm for $CDCl_3$ and 3.31 ppm for CD_3OD); ^{13}C NMR: 77.00 ppm for $CDCl_3$ and 49.00 ppm for CD_3OD). ^{19}F spectra were referenced to $CFCl_3$ as the internal reference which was set at δ 0.00 ppm. Carbon substitution degrees were established by DEPT pulse sequences. Abbreviations used for NMR signals are as follows: s =singlet, d = doublet, dd = double doublet, ddd = doublet of double doublet, t = triplet, dt = double triplet, ddt = double doublet of triplets, q = quadruplet, br = broad, quint = quintuplet, m = multiplet, BiPh = Biphenyl ring, Pz = Pyrazol ring. High-resolution mass spectra (HRMS) were run by the electrospray (ES) mode, which was obtained with a Q-TOF premier mass spectrometer with an electrospray source (Waters, Manchester, UK).

Sephadex G-25 HiTrap Desalting columns from GE Healthcare (Uppsala, Sweden) were utilized for protein–hapten conjugate purification. Hybridoma fusion and cloning supplement was obtained from Roche Applied Science (Mannheim, Germany). P3-X63-Ag 8.653 mouse plasmacytoma cell line was acquired from the European Collection of Cell Cultures (Salisbury, UK). Cell culture media (high-glucose Dulbecco's modified Eagle's medium), gentamicin solution, and hypoxanthine–thymidine and hypoxanthine–aminopterine–thymidine supplements were purchased from Gibco BRL (Paisley, UK). Poly(ethylene glycol) (PEG1500), fetal bovine serum, 200 mM alanyl–glutamine solution, red blood cell lysing buffer Hybri-Max, MEM non-essential amino acid solution, Freund's adjuvants, and *o*-phenylenediamine, and triphenylphosphate were obtained from Merck (Madrid, Spain). HiTrap protein G HP columns for mouse IgG purification were procured from General Electric Healthcare (Uppsala, Sweden). Rabbit anti-mouse immunoglobulin polyclonal antibody conjugated to peroxidase was from Dako (Glostrup, Denmark). Primary/secondary amine from Varian (Palo Alto, CA) and organic solvents from Scharlab (Barcelona, Spain) were used for sample preparation. Hapten density of protein conjugates was determined with a 5800 matrix-assisted laser desorption ionization time-

of-flight (MALDI-TOF/TOF) mass spectrometry apparatus from ABSciex (Framingham, MA). Costar flat-bottom high-binding 96-well polystyrene ELISA plates were from Corning (Corning, NY). ELISA absorbances were read with a PowerWave HT from BioTek Instruments (Winooski, VT). Microwells were washed with an ELx405 microplate washer also from BioTek Instruments. Fluxapyroxad residues were determined by HPLC using a UPLC Acquity system from Waters (Milford, MA) furnished with a binary solvent delivery system, an autosampler, and a BEH C18 (1.7 µm, 2.1 × 50 mm) column also from Waters. An Acquity triple quadrupole MS detector, also from Waters, with a Z-spray electrospray ionization source (3.5 kV capillary voltage, and 120 °C and 300 °C source and desolvation temperature, respectively) were employed for tandem mass acquisitions.

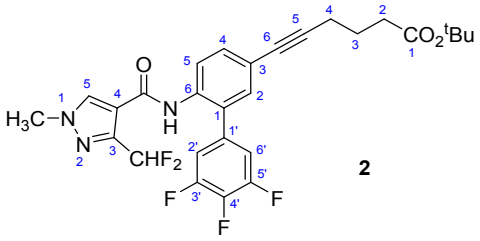
Synthesis of haptens

Spectrometric data of intermediates of the synthesis of hapten FX*b*



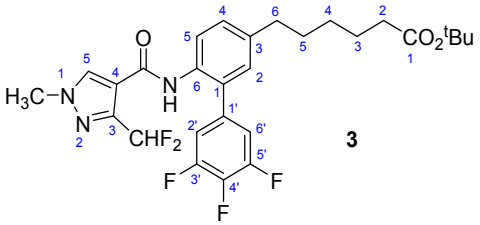
1

3-(Difluoromethyl)-1-methyl-N-(3',4',5'-trifluoro-5-iodo-[1,1'-biphenyl]-2-yl)-1H-pyrazole-4-carboxamide (**1**). Mp 181.1-182.6 °C (from hexane-Et_2O); IR (neat) ν_{max} (cm^{-1}) 3416 (m), 3139 (w), 3063 (w), 1663 (s), 1542 (s), 1519 (s), 1393 (s), 1038 (s), 763 (m); ^1H NMR (300 MHz, $CDCl_3$) δ (ppm) 8.06 (1H, d, *J* = 8.7 Hz, H-3 BiPh), 7.97 (1H, s, H-5 Pz), 7.78 (1H, br s, NH), 7.72 (1H, dd, *J* = 8.7, 2.0 Hz, H-4 BiPh), 7.54 (1H, d, *J* = 2.0 Hz, H-6 BiPh), 6.97 (2H, m, H-2' and H-6' BiPh), 6.60 (1H, t, *J* = 54.2 Hz, CHF_2), 3.92 (3H, s, NMe); ^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm) 159.2 (CONH), 151.3 (ddd, *J* = 252.1, 10.0, 4.2 Hz, C-3' and C-5' BiPh), 142.0 (t, *J* = 29.6 Hz, C-3 Pz), 139.8 (dt, *J* = 253.6, 15.1 Hz, C-4' BiPh), 138.4 (C-4 BiPh), 138.1 (C-5 Pz), 136.6 (C-6 BiPh), 134.7 (C-2 BiPh), 132.6 (C-1 BiPh), 132.3 (td, *J* = 8.1, 5.0 Hz, C-1' BiPh), 124.6 (C-3 BiPh), 116.4 (C-4 Pz), 113.8 (dd, *J* = 14.6, 7.2 Hz, C-2' and C-6' BiPh), 111.7 (t, *J* = 232.7 Hz, CHF_2), 88.3 (C-5 BiPh), 39.5 (NMe); HRMS (TOF MS ES+) *m/z* calcd for $C_{18}H_{12}F_5IN_3O$ $[M+H]^+$ 507.9940, found 507.9933.



2

tert-Butyl 6-(6-(3-(difluoromethyl)-1-methyl-1H-pyrazole-4-carboxamido)-3',4',5'-trifluoro-[1,1'-biphenyl]-3-yl)hex-5-ynoate (**2**). Mp 104-105.5 °C (from hexane-Et_2O); IR (neat) ν_{max} (cm^{-1}) 3421 (m), 3283 (s), 3124 (m), 2979 (s), 2233 (w), 1724 (s), 1660 (s) 1532 (s), 1149 (s), 1044 (s), 861 (m), 653 (m); ^1H NMR (300 MHz, $CDCl_3$) δ (ppm) 8.18 (1H, d, *J* = 8.6 Hz, H-5 BiPh), 7.93 (1H, s, H-5 Pz), 7.82 (1H, t, *J* = 4.0 Hz, NH), 7.42 (1H, dd, *J* = 8.5, 1.9 Hz, H-4 BiPh), 7.23 (1H, d, *J* = 1.9 Hz, H-2 BiPh), 6.97 (2H, m, H-2' and H-6' BiPh), 6.63 (1H, t, *J* = 54.2 Hz, CHF_2), 3.89 (3H, s, NMe), 2.45 (2H, t, *J* = 7.0 Hz, H-4), 2.38 (2H, t, *J* = 7.4 Hz, H-2), 1.87 (4H, quint, *J* = 7.3 Hz, H-3 and H-4), 1.44 (9H, s, CMe_3); ^{19}F NMR (282 MHz, $CDCl_3$) δ (ppm) -109.4 (2F, s, CHF_2), -133.9 (2F, d, *J* = 20.5 Hz, F-3' and F-5' BiPh), -161.5 (1F, t, *J* = 20.5, Hz, F-4' BiPh); ^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm) 172.4 (CO_2), 159.2 (CONH), 151.4 (ddd, *J* = 251.6, 10.0, 4.0 Hz, C-3' and C-5' BiPh), 142.1 (t, *J* = 29.7 Hz, C-3 Pz), 139.6 (dt, *J* = 253.0, 15.1 Hz, C-4' BiPh), 136.2 (C-5 Pz), 134.0 (C-6 BiPh), 133.2 (td, *J* = 8.1, 4.9 Hz, C-1' BiPh), 133.0 (C-2 BiPh), 132.2 (C-4 BiPh), 130.6 (C-1 BiPh), 122.6 (C-5 BiPh), 120.5 (C-3 BiPh), 116.4 (C-4 Pz), 113.7 (dd, *J* = 14.6, 7.0 Hz, C-2' and C-6' BiPh), 111.5 (t, *J* = 232.4 Hz, CHF_2), 89.8 (C-5), 80.3 (*C*Me_3), 80.2 (C-6), 39.5 (NMe), 34.4 (C-2), 28.1 (C*Me*$_3$), 24.0 (C-3), 18.8 (C-4); HRMS (TOF MS ES+) *m/z* calcd for $C_{28}H_{27}F_5N_3O_3$ $[M+H]^+$ 548.1967, found 548.1943.



3

tert-Butyl 6-(6-(3-(difluoromethyl)-1-methyl-1H-pyrazole-4-carboxamido)-3',4',5'-trifluoro-[1,1'-biphenyl]-3-yl)hexanoate (**3**). IR (neat) ν_{max} (cm^{-1}) 3432 (w), 3293 (m), 3119 (w), 2970 (w), 2930 (s), 1721 (s), 1634 (s), 1531 (s), 1368 (s), 1158 (s), 1044 (s), 856 (m); ^1H NMR (300 MHz, $CDCl_3$) δ (ppm) 8.02 (1H, d, *J* = 8.3 Hz, H-5 BiPh), 7.94 (1H, s, H-5 Pz), 7.76 (1H, t, *J* = 4.1 Hz, NH), 7.23 (1H, dd, *J* = 8.4, 2.1 Hz, H-4 BiPh), 7.02 (1H, d, *J* = 2.1 Hz, H-2 BiPh), 6.99 (2H, m, H-2' and H-6' BiPh), 6.65 (1H, t, *J* = 54.2 Hz, CHF_2), 3.91 (3H, s, NMe), 2.61 (2H, t, *J* = 7.5 Hz, H-6), 2.21 (2H, t, *J* = 7.4 Hz, H-2), 1.61 (4H, m, H-3 and H-5), 1.43 (9H, s, CMe_3), 1.36 (2H, m, H-4); ^{19}F NMR (282 MHz, $CDCl_3$) δ (ppm) -109.4 (2F, s, CHF_2), -134.4 (2F, d, *J* = 20.6 Hz, F-3' and F-5' BiPh), -162.3 (1F, t, *J* = 20.6 Hz, F-4' BiPh); ^{13}C NMR (75

MHz, $CDCl_3$) δ (ppm) 173.1 (CO_2), 159.4 (CONH), 151.3 (ddd, *J* = 250.9, 9.7, 4.1 Hz, C-3' and C-5' BiPh), 142.3 (t, *J* = 29.1 Hz, C-3 Pz), 139.9 (C-6 BiPh), 139.4 (dt, *J* = 252.3, 15.1 Hz, C-4' BiPh), 136.0 (C-5 Pz), 134.3 (td, *J* = 7.9, 4.9 Hz, C-1' BiPh), 132.0 (C-3 BiPh), 131.4 (C-1 BiPh),129.8 (C-2 BiPh), 129.1 (C-4 BiPh), 123.7(C-5 BiPh), 116.6 (C-4 Pz), 113.6 (dd, *J* = 14.6, 6.9 Hz, C-2' and C-6' BiPh), 111.6 (t, *J* = 232.9 Hz, CHF_2), 80.0 (CMe_3), 39.5 (NMe), 35.4 (C-6), 35.1 (C-2), 31.0 (C-3), 28.6 (C-5), 28.1 (CMe_3), 24.84 (C-4); HRMS (TOF MS ES+) *m/z* calcd for $C_{28}H_{31}F_5N_3O_3$ $[M+H]^+$ 552.2280, found 552.2253.

Synthesis of hapten FX*h*

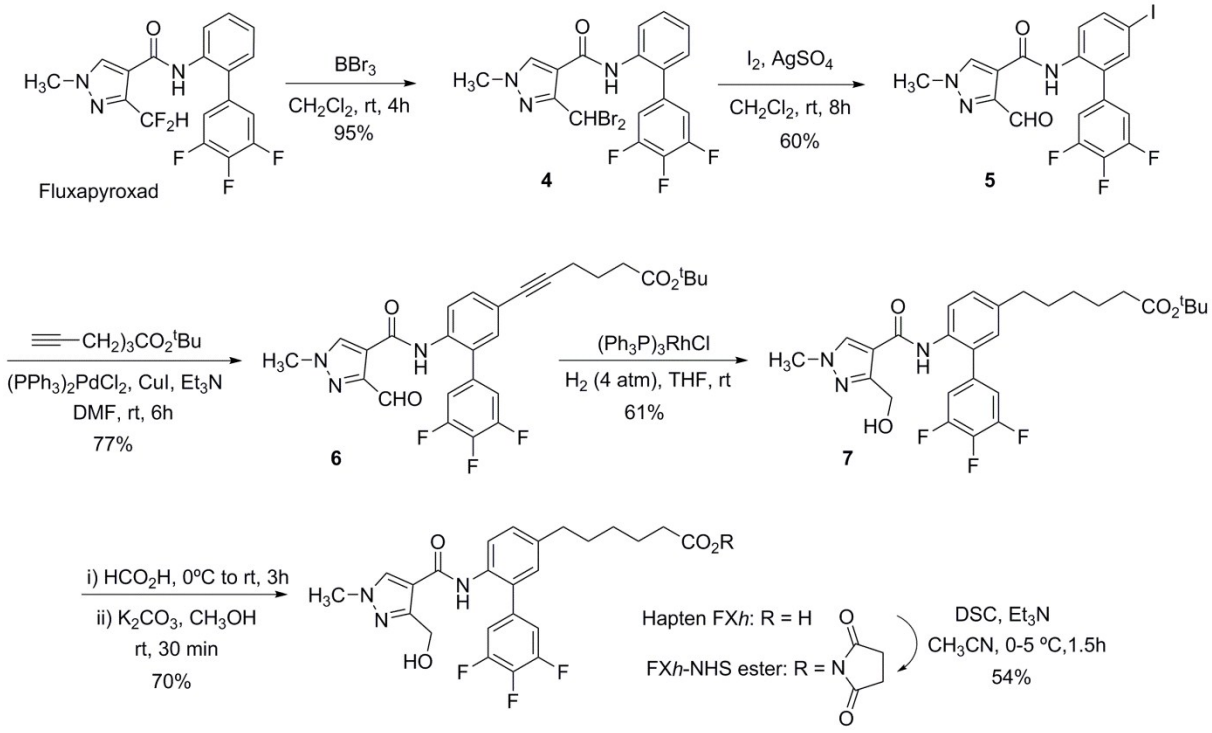
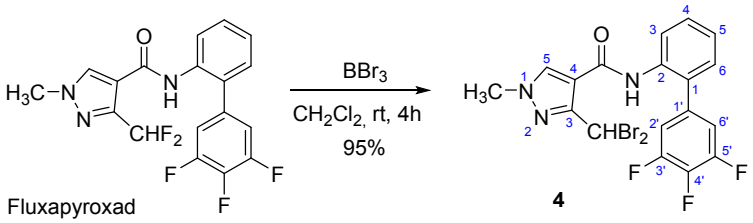
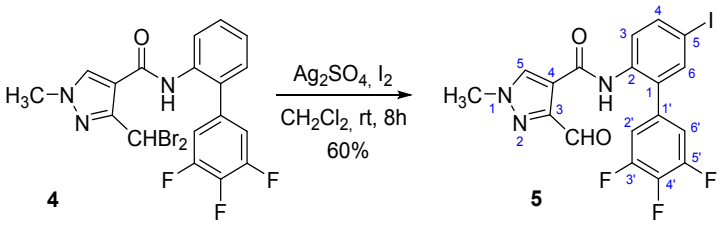


Figure S1. Schematic representation of the synthesis and activation of hapten FX*h*.



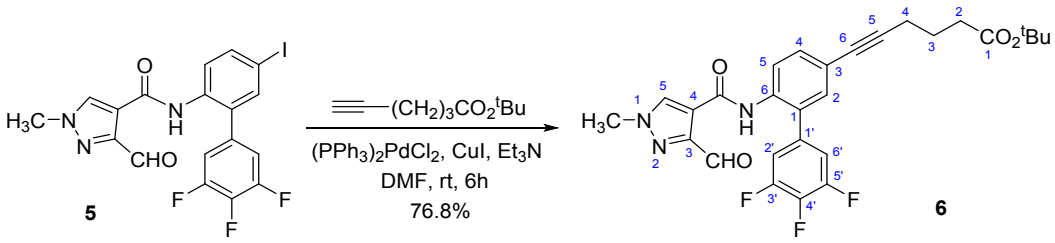
Synthesis of 3-(dibromomethyl)-1-methyl-N-(3',4',5'-trifluoro-[1,1'-biphenyl]-2-yl)-1H-pyrazole-4-carboxamide (**4**). A 1M solution of BBr_3 in CH_2Cl_2 (3.15 mL, 3.15 mmol, 6 equiv) was dropwise added to a solution of fluoxapyroxad (200 mg, 0.525 mmol) in anhydrous CH_2Cl_2 (8 mL) at -78 °C under nitrogen. The reaction mixture was allowed to warm slowly to room temperature and was stirred for

4 h. The mixture was then cooled to 0 °C, then carefully quenched with water and extracted with CH_2Cl_2. The combined organic extracts were washed with brine, dried over anhydrous $MgSO_4$ and concentrated under reduced pressure to give dibromide **4** (251 mg, 95.1%) as a solid, which was deemed sufficiently pure to be used in the next step without any further purification. Mp 198.6-199.4 °C (crystals obtained from slow evaporation from a CH_2Cl_2 solution); IR (neat) ν_{max} (cm^{-1}) 3213 (s), 3117 (w), 3039 (w), 1640 (s), 1541 (s), 1532 (s), 1516 (s), 1492 (s), 1042 (s), 760 (s); ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm) 8.09 (1H, br d, *J* = 7.8 Hz, H-3 BiPh), 7.54 (1H, br s, NH), 7.52 (1H, s, H-5 Pz), 7.43 (1H, ddd, *J* = 8.1, 6.3, 2.9 Hz, H-4 BiPh), 7.29-7.23 (2H, m, H-5 and H-6 BiPh), 7.19 (1H, s, $CHBr_2$), 7.10-6.99 (2H, m, H-2' and H-6' BiPh), 3.93 (3H, s, NMe); ^{19}F NMR (282 MHz, $CDCl_3$) δ (ppm) -133.2 (2F, d, *J* = 20.6 Hz, F-3' and F-5' BiPh), -161.1 (1F, t, *J* = 20.6 Hz, F-4' BiPh);^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm) 160.3 (CONH), 151.4 (ddd, *J* = 251.7, 10.0, 4.1 Hz, C-3' and C-5' BiPh), 151.3 (C-3 Pz), 139.5 (dt, *J* = 253.5, 15.1 Hz, C-4' BiPh), 134.4 (td, *J* = 7.8, 5.0 Hz, C-1' BiPh), 133.8 (C-2 BiPh), 131.7 (C-5 Pz), 131.1 (C-1 BiPh), 130.1 (C-6 BiPh), 129.4 (C-4 BiPh), 125.7 (C-5 BiPh), 123.9 (C-3 BiPh), 113.6 (C-4 Pz), 113.6 (dd, *J* = 14.5, 7.0 Hz, C-2' and C-6' BiPh), 39.9 (NMe), 30.1 ($CHBr_2$); HRMS (TOF MS ES+) *m/z* calcd for $C_{18}H_{13}{}^{79}Br_2F_3N_3O$ $[M+H]^+$ 501.9372, found 501.9377.

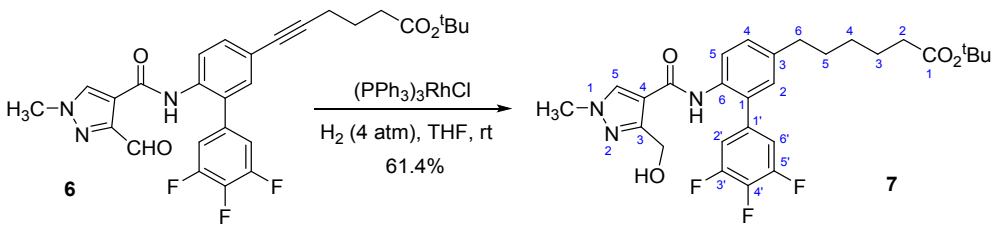


Synthesis of 3-formyl-1-methyl-N-(3',4',5'-trifluoro-5-iodo-[1,1'-biphenyl]-2-yl)-1H-pyrazole-4-carboxamide (**5**). Ag_2SO_4 (104.6 mg, 0.335 mmol, 1.5 equiv) was added in portion-wise to a stirred solution of dibromide **4** (112.6 mg, 0.224 mmol) and iodine (94.8 mg, 0.375 mmol, 1.7 equiv) in CH_2Cl_2 (1.5 mL). The reaction mixture was stirred in the dark at room temperature for 22 hours, diluted with CH_2Cl_2 and filtered through cotton wool plug to separate the yellow precipitate formed. The filtrate was washed with an aqueous solution of sodium bisulfite and brine, dried over anhydrous Na_2SO_4 and concentrated under reduced pressure to give a residue that was chromatographed on silica gel to obtain pure iodo-aldehyde **5** (65.6 mg, 60.1%) as an amorphous solid. IR (neat) ν_{max} (cm^{-1}) 3251 (w), 3124 (w), 3070 (w), 2915 (w), 2851 (w), 1688 (s), 1654 (s), 1611 (m), 1585 (m), 1555 (m), 1533 (s), 1035 (s), 783 (m), 764 (m); ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm) 10.65 (1H, br s, NH), 9.69 (1H, d, *J* = 0.6, CHO), 8.15 (1H, s, H-5 Pz), 7.89 (1H, d, *J* = 8.7 Hz, H-3 BiPh), 7.71 (1H, dd, *J* = 8.7, 2.1 Hz, H-4 BiPh), 7.59 (1H, d, *J* = 2.1 Hz, H-6 BiPh), 7.02 (2H, m, H-2' and H-6' BiPh), 4.04 (3H, s, NMe); ^{19}F NMR (282 MHz, $CDCl_3$) δ (ppm) -134.4 (2F, d, *J* = 20.6 Hz, F-3' and F-5' BiPh), -161.7 (1F, t, *J* = 20.6 Hz, F-4'

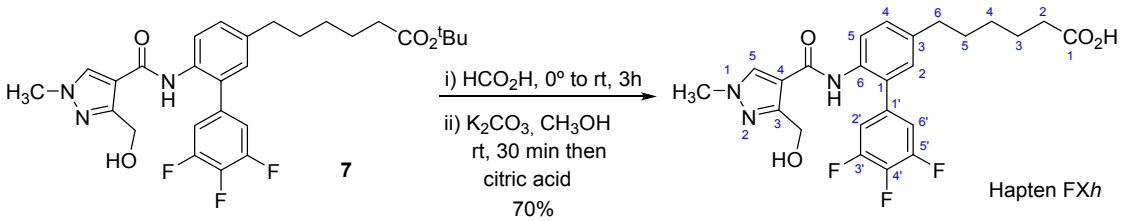
BiPh); ^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm) 188.5 (CHO), 159.0 (CONH), 151.2 (ddd, *J* = 250.3, 10.3, 4.3 Hz, C-3' and C-5' BiPh), 145.7 (C-3 Pz), 132.3 (td, *J* = 8.1, 5.0 Hz, C-1' BiPh), 126.2(C-3 BiPh), 113.8 (dd, *J* = 14.7, 6.9 Hz, C-2' and C-6' BiPh), 120.4 (C-4 Pz), 89.0(C-5 BiPh), 40.2 (NMe); HRMS (TOF MS ES+) *m/z* calcd for $C_{18}H_{12}F_3IN_3O_2$ $[M+H]^+$ 485.9921, found 485.9917.



Synthesis of tert-butyl 6-(3',4',5'-trifluoro-6-(3-formyl-1-methyl-1H-pyrazole-4-carboxamido)-[1,1'-biphenyl]-3-yl)hex-5-ynoate (**6**). Et_3N (0.150 mL) was added to a mixture of iodide **5** (59.6 mg, 0.123 mmol), CuI (1.6 mg, 0.008 mmol, 0.07 equiv), $(PPh_3)_2PdCl_2$ (2.6 mg, 0.004 mmol, 0.03 equiv) and *tert*-butyl hex-5-ynoate (25 mg, 0.148 mmol, 1.2 equiv) in dry DMF (0.150 mL) under nitrogen at room temperature. The reaction mixture was stirred at room temperature for 6 hours, quenched with water and extracted with EtOAc. The extracts were washed with an aqueous LiCl solution and brine and dried over anhydrous $MgSO_4$. Chromatographic purification, using hexane-EtOAc mixtures from 9:1 to 1:1 as eluent, afforded acetylenic compound **6** (49.7 mg, 76.8%) as an amorphous solid. IR (neat) ν_{max} (cm^{-1}) 3125 (m), 2977 (w), 2929 (w), 1732 (s), 1678 (s), 1655 (s), 1615 (m), 1589 (s), 1541 (s), 1301 (m), 1149 (s), 1041 (s), 189 (m); ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm) 10.65 (1H, br s, NH), 9.69 (1H, d, *J* = 0.7 Hz, CHO), 8.15 (1H, d, *J* = 0.6 Hz, H-5 Pz), 8.07 (1H, d, *J* = 8.5 Hz, H-5 BiPh), 7.42 (1H, dd, *J* = 8.5, 2.0 Hz, H-4 BiPh), 7.29 (1H, d, *J* = 2.0 Hz, H-2 BiPh), 7.02 (2H, m, H-2' and H-6' BiPh), 4.03 (3H, s, NMe), 2.46 (2H, t, *J* = 7.0 Hz, H-4), 2.40 (2H, t, *J* = 7.3 Hz, H-2), 1.88 (4H, quint, *J* = 7.4 Hz, H-3 and H-4), 1.45 (9H, s, CMe_3); ^{19}F NMR (282 MHz, $CDCl_3$) δ (ppm) -134.8 (2F, d, *J* = 20.6 Hz, F-3' and F-5' BiPh), -162.3 (1F, t, *J* = 20.6, Hz, F-4' BiPh); ^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm) 188.4 (CHO), 172.4 (CO_2), 158.9 (CONH), 151.1 (ddd, *J* = 251.1, 10.0, 4.4 Hz, C-3' and C-5' BiPh), 145.6 (C-3 Pz),138.1 (C-5 Pz), 139.5 (dt, *J* = 252.0, 15.2 Hz, C-4' BiPh), 134.3 (C-6 BiPh), 134.1 (td, *J* = 8.1, 5.0 Hz, C-1' BiPh), 133.1 (C-2 BiPh), 132.0 (C-4 BiPh), 131.8 (C-1 BiPh), 124.1 (C-5 BiPh), 120.7 (C-3 BiPh), 120.4 (C-4 Pz), 113.8 (dd, *J* = 14.7, 6.8 Hz, C-2' and C-6' BiPh), 89.7 (C-5), 80.4 (*C*Me_3), 80.3 (C-6), 40.1 (NMe), 34.4 (C-2), 28.1 (C*Me*$_3$), 24.0 (C-3), 18.8 (C-4); HRMS (TOF MS ES+) *m/z* calcd for $C_{28}H_{27}F_3N_3O_4$ $[M+H]^+$ 526.1948, found 526.1944.



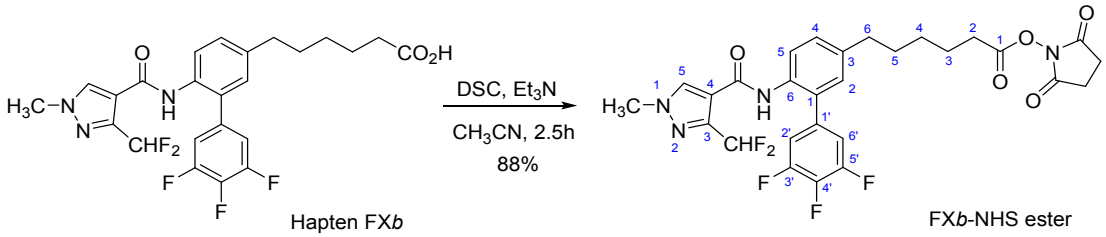
Synthesis of tert-butyl 6-(3',4',5'-trifluoro-6-(3-(hydroxymethyl)-1-methyl-1H-pyrazole-4-carboxamido)-[1,1'-biphenyl]-3-yl)hexanoate (**7**). A solution of alkyne **6** (45.1 mg, 0.086 mmol) and $(Ph_3P)_3RhCl$ (5 mg, 0.005 mmol, 0.06 equiv) in anhydrous THF (500 µL) was stirred under a hydrogen atmosphere of 60 psi at room temperature for 3 days. Chromatographic purification of the residue obtained after evaporation of the solvent, using hexane-EtOAc mixtures from 7:3 to 3:7 as eluent, gave, in order of elution, the aldehyde resulting from the reduction of only the triple bond (17 mg) followed by the product of reduction of both the carbonyl and triple bonds, compound **7** (27.9 mg, 61.4%). Mp 125.8-126.4 °C (from hexane-EtOAc-$CHCl_3$); IR (neat) ν_{max} (cm^{-1}) 3277 (br, m), 3237 (m), 3133 (m), 2923 (s), 2857 (m), 1734 (s), 1656 (s), 1615 (m), 1593 (m), 1547 (s), 1559 (s), 1425 (m), 1164 (s), 1042 (s); ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm) 8.81 (1H, br s, NH), 7.86 (1H, d, *J* = 8.4 Hz, H-5 BiPh), 7.75 (1H, s, H-5 Pz), 7.22 (1H, dd, *J* = 8.4, 2.1 Hz, H-4 BiPh), 7.03 (3H, m, H-2, H-2' and H-6' BiPh), 4.56 (2H. d, *J* = 6.0 Hz, CH_2O), 3.82 (3H, s, NMe), 3.41 (1H, d, *J* = 6.0 Hz, OH), 2.61 (2H, t, *J* = 7.5 Hz, H-6), 2.20 (2H, t, *J* = 7.2 Hz, H-2), 1.68-1.56 (4H, m, H-3 and H-5), 1.43 (9H, s, CMe_3), 1.36 (2H, m, H-4); ^{19}F NMR (282 MHz, $CDCl_3$) δ (ppm) -134.4 (2F, d, *J* = 20.6 Hz, F-3' and F-5' BiPh), -162.2 (1F, t, *J* = 20.6 Hz, F-4' BiPh); ^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm) 173.3 (CO_2), 161.9 (CONH), 151.0 (ddd, *J* = 251.0, 10.0, 4.0 Hz, C-3' and C-5' BiPh), 150.4 (C-3 Pz), 140.2 (C-6 BiPh), 135.2 (td, *J* = 8.0, 5.0 Hz, C-1' BiPh), 134.5 (C-5 Pz), 132.3 (C-3 BiPh), 132.2 (C-1 BiPh), 130.0 (C-2 BiPh), 129.2 (C-4 BiPh), 125.0 (C-5 BiPh), 117.4 (C-4 Pz), 113.7 (dd, *J* = 14.8, 6.7 Hz, C-2' and C-6' BiPh), 80.2 (*C*Me_3), 58.1 (CH_2OH), 39.1 (NMe), 35.6 (C-6), 35.3 (C-2), 31.2 (C-3), 28.8 (C-5), 28.2 (C*Me*$_3$), 25.0 (C-4); HRMS (TOF MS ES+) *m/z* calcd for $C_{28}H_{33}F_3N_3O_4$ $[M+H]^+$ 532.2418, found 532.2409.



Synthesis of 6-(3',4',5'-trifluoro-6-(3-(hydroxymethyl)-1-methyl-1H-pyrazole-4-carboxamido)-[1,1'-biphenyl]-3-yl)hexanoic acid (Hapten FX*h*). A solution of *tert*-butyl ester **7** (46.5 mg, 0.087 mmol) in HCO_2H (1 mL) was stirred under anhydrous conditions at 0 °C for 1 hour and then at room

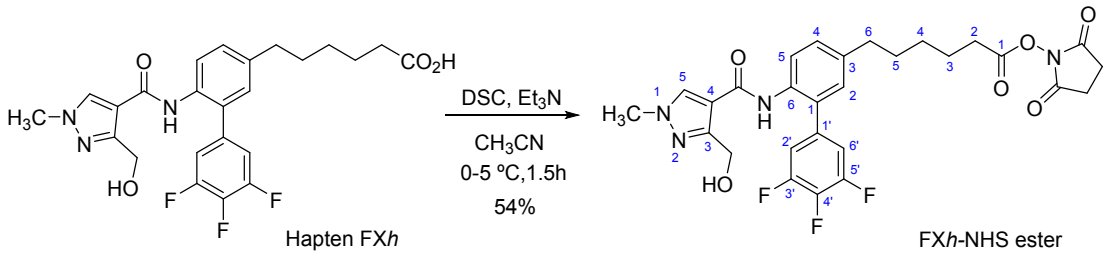
temperature for an additional 2 hours. The mixture was concentrated under vacuum and stripped with toluene to removal residual formic acid. The obtained residue (as shown by ^{1}H NMR spectroscopy, a mixture of hapten FX and the corresponding *O*-formylated derivative) was dissolved in a solution of K_2CO_3 (13.5 mg, 0.098 mmol) in MeOH (2 mL) and the mixture was stirred at room temperature for 30 minutes, then cooled in an ice-water bath and acidified with citric acid. The residue left after evaporation of the solvent was dissolved in EtOAc, washed with water and brine, dried over anhydrous Na_2SO_4 and concentrated under vacuum. Chromatographic purification of the crude product obtained, using $CHCl_3$-MeOH from 100:0 to 95:5 as eluent, afforded hapten FX*h* (29.1 mg, 70%). Mp 168.6-169.3 °C (from $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm), 8.91 (1H, br s, NH), 7.81 (1H, d, *J* = 8.3 Hz, H-5 BiPh), 7.77 (1H, s, H-5 Pz), 7.21 (1H, dd, *J* = 8.3, 1.8 Hz, H-4 BiPh), 7.05-6.97 (3H, m, H-2, H-2' and H-6' BiPh), 4.56 (2H, s, CH_2O), 3.82 (3H, s, NMe), 2.62 (2H, t, *J* = 7.5 Hz, H-6), 2.33 (2H, t, *J* = 7.3 Hz, H-2), 1.65 (4H, m, H-3 and H-5), 1.41 (2H, m, H-4); ^{19}F NMR (282 MHz, $CDCl_3$) δ (ppm) –134.5(2F, d, *J* = 20.0 Hz, F-3' and F-5' BiPh), -162.3 (1F, t, *J* = 20.0 Hz, F-4' BiPh); ^{13}C NMR (75 MHz, CD_3OD) δ (ppm) 177.6 (CO_2), 164.5 (CONH), 152.3 (ddd, *J* = 248.4, 9.9, 4.1 Hz, C-3' and C-5' BiPh), 152.1 (C-3 Pz), 142.5 (C-6 BiPh), 140.3 (dt, *J* = 250.1, 14.9 Hz, C-4' BiPh), 137.4 (dt, *J* = (8.3, 5.3 Hz, C-1' BiPh), 136.2 (C-5 Pz), 135.7 (C-3 BiPh), 133.2 (C-1 BiPh), 131.2 (C-2 BiPh), 130.1 (C-4 BiPh), 127.8 (C-5 BiPh), 117.5 (C-4 Pz), 114.5 (dd, *J* = 14.8, 6.6 Hz, C-2' and C-6' BiPh), 58.3 (CH_2O), 39.1 (NMe), 36.1 (C-6), 34.9 (C-2), 32.2 (C-5), 29.8 (C-4), 25.9 (C-3); HRMS (TOF MS ES+) *m/z* calcd for $C_{24}H_{25}F_3N_3O_4$ $[M+H]^+$ 476.1792, found 476.1809.

Hapten activation: preparation of *N*-hydroxysuccinimidyl esters



Synthesis of 2,5-dioxopyrrolidin-1-yl 6-(6-(3-(difluoromethyl)-1-methyl-1H-pyrazole-4-carboxamido)-3',4',5'-trifluoro-[1,1'-biphenyl]-3-yl)hexanoate (FX*b*-NHS ester). Hapten FX*b* (23.5 mg, 0.047 mmol) and *N,N'*-disuccinimidyl carbonate (15.65 mg, 0.0611 mmol, 1.3 equiv) were dissolved in anhydrous acetonitrile (0.6 mL) under nitrogen in an ice-water bath. Et_3N (25 µL, 0.179 mmol, 3.8 equiv) was them added and the resulting mixture was stirred at room temperature until complete consumption of starting material (as observed by thin-layer chromatography using $CHCl_3$:EtOH 95:5 as eluent, about 2.5 hours). The reaction mixture was diluted with $CHCl_3$, washed with a 10%

aqueous solution of $NaHCO_3$ and brine, dried over anhydrous Na_2SO_4 and concentrated under vacuum to give an oily residue that was filtered over a short pad of silica gel, eluting with $CHCl_3$, to afford nearly pure FX*b*-NHS *e*ster (24.6 mg, 87.7%), as determined by the 'H NMR spectra. ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm) 8.01 (1H, d, *J* = 8.4 Hz, H-5 BiPh), 7.94 (1H, s, H-5 Pz), 7.77 (1H, br t, *J* = 4.2 Hz, NH), 7.23 (1H, dd, *J* = 8.4, 2.4 Hz, H-4 BiPh), 7.03 (1H, d, *J* = 2.4 Hz, H-2 BiPh), 7.00 (2H, m, H-2' and H-6' BiPh), 6.66 (1H, t, *J* = 54.3 Hz, CHF_2), 3.91 (3H, s, NMe), 2.83 (4H, br s, $COCH_2CH_2CO$), 2.63 (2H, t, *J* = 7.5 Hz, H-6), 2.60 (2H, t, *J* = 7.5 Hz, H-2), 1.78 (2H, quint, *J* = 7.5 Hz, H-5), 1.66 (2H, quint, *J* = 7.5 Hz, H-3), 1.47 (2H, m, H-4).



Synthesis of 2,5-dioxopyrrolidin-1-yl 6-(3',4',5'-trifluoro-6-(3-(hydroxymethyl)-1-methyl-1H-pyrazole-4-carboxamido)-[1,1'-biphenyl]-3-yl)hexanoate (FX*h*-NHS ester). Et_3N (14 µL, 0.098 mmol, 3.8 equiv) was added to an ice-water bath cooled solution of hapten FX*h* (12.3 mg, 0.026 mmol) and *N,N'*-disuccinimidyl carbonate (8.0 mg, 0.031 mmol, 1.2 equiv) in anhydrous acetonitrile (500 µL) under nitrogen. The mixture was stirred at the same temperature for 1.5 hours and then diluted with EtOAc and washed with a 10% aqueous solution of $NaHCO_3$ and brine and dried over anhydrous Na_2SO_4. Purification of the residue left after evaporation of the solvent by preparative thin layer chromatography (PTLC), using $CHCl_3$-MeOH 95:5 as eluent, afforded FX*h*-NHS ester (8.0 mg, 54%). ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm) 8.76 (1H, br s, NH), 7.89 (1H, d, *J* = 8.3 Hz, H-5 BiPh), 7.77 (1H, s, H-5 Pz), 7.23 (1H, dd, *J* = 8.3, 2.1 Hz, H-4 BiPh), 7.07-7.01 (3H, m, H-2, H-2' and H-6' BiPh), 6.60 (2H, s, CH_2O), 3.85 (3H, s, NMe), 2.83 (4H, br s, $COCH_2CH_2CO$), 2.63 (2H, t, *J* = 7.5 Hz, H-6), 2.61 (2H, t, *J* = 7.4 Hz, H-2), 1.78 (2H, quint, *J* = 7.5 Hz, H-5), 1.67 (2H, m, H-3), 1.47 (2H, m, H-4).

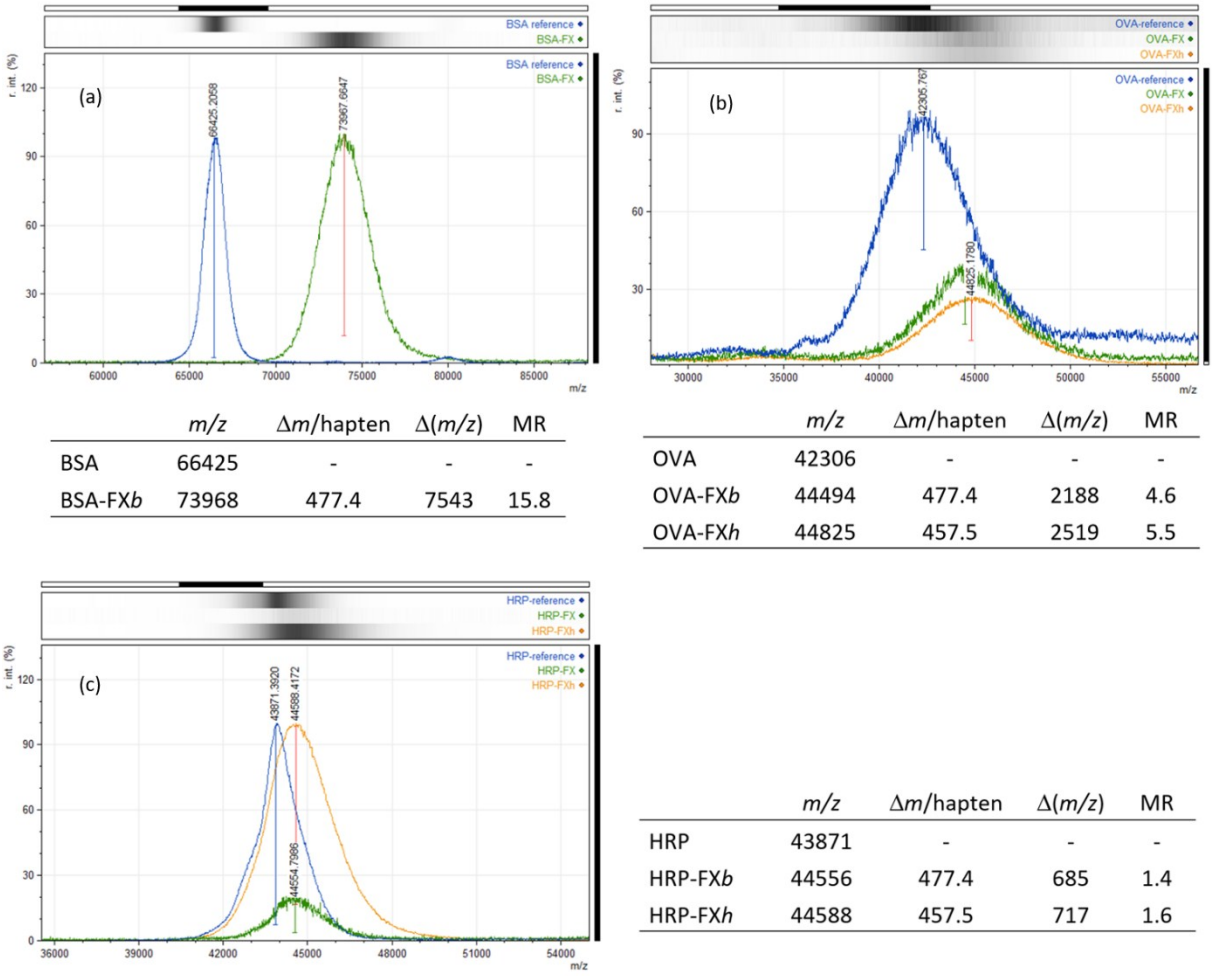
MALDI mass spectrometry analysis of immunizing and assay bioconjugates

Sample preparation. 100 µL of each of the protein conjugate solutions (0.5-1 mg/mL) were dialyzed against milliQ water and then freeze-dried and lyophilized. The samples were dissolved in MilliQ H_2O to theoretical final concentration 1 µg/µL. Then, 1 µL of every sample solution was spotted onto the MALDI plate. After the droplets were air dried at room temperature, 1 µL of matrix

(10 mg/mL sinapinic acid (Bruker) in 0.1% trifluoroacetic acid-CH_3CN/H_2O (7:3 v/v) was added and allowed to air-dry at room temperature.

Mass spectrometry analysis. The resulting mixtures were analyzed in a MALDI-TOF/TOF apparatus in positive linear mode (1500 shots every position) in a mass range of 10000-100000 m/z. Previously, the plate was calibrated with 1 µL of the TOF/TOF calibration mixture (ABSciex), in 13 positions. Every sample was calibrated by 'close external calibration' method with a BSA, OVA or HRP spectrum acquired in a close position.

As determined by MALDI-TOF, the bioconjugates prepared showed the final molar ratios (MR) showed in Figure S2.



	m/z	Δ*m*/hapten	Δ(*m/z*)	MR
BSA	66425	-	-	-
BSA-FX*b*	73968	477.4	7543	15.8

	m/z	Δ*m*/hapten	Δ(*m/z*)	MR
OVA	42306	-	-	-
OVA-FX*b*	44494	477.4	2188	4.6
OVA-FX*h*	44825	457.5	2519	5.5

	m/z	Δ*m*/hapten	Δ(*m/z*)	MR
HRP	43871	-	-	-
HRP-FX*b*	44556	477.4	685	1.4
HRP-FX*h*	44588	457.5	717	1.6

Figure S2. MALDI-TOF-MS spectra of proteins (blue) and the corresponding conjugates with haptens FX*b* (green) and FX*h* (orange): (a) BSA conjugates; (b) OVA conjugates; (c) HRP conjugates

Antibody generation

Two groups of four BALB/c female mice each (8–10 weeks old) were immunized by intraperitoneal injections; one group with BSA–FX*n* and the other group with BSA–FX*b*. Each BSA-hapten conjugate solution was emulsified with one volume 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. Each mouse received 100 μg per boost of immunizing conjugate in 200 μL of emulsion. After a resting period of at least three weeks, mice received an intraperitoneal booster injection of 100 μg of immunogen in 200 μL of sterile PBS four days before cell fusion.

After cell fusion, a two-step screening procedure was followed in order to identify those hybridomas that produced high-affinity binders. Twelve days after fusion, hybridoma culture supernatants were first screened by differential competitive ELISA on microtiter plates coated with 0.1 μg/mL (100 μL per well) of the homologous OVA–hapten conjugate. 50 μL of each supernatant was added to two adjacent wells of an ELISA plate, one containing 50 μL of PBS (blank) and the other containing 50 μL of 200 nM fluxapyroxad in PBS. The signal ratio in both wells was used as the criterion for selecting the antibodies with the highest affinity. Hybridoma supernatants affording signals higher than 3.0 in the absence of fungicide and those already showing high-affinity to fluxapyroxad received fresh culture medium and they were reevaluated on next day by checkerboard indirect competitive ELISA. Each supernatant was assayed at four dilutions (1/8, 1/32, 1/128, and 1/512) in ELISA plates coated with two coating concentrations of the homologous OVA–hapten conjugate (0.01 and 0.1 μg/mL) and using three fluxapyroxad levels (0, 10, and 100 nM).

Specificity of mAbs

Table S1. Cross-reactivity values (%).

mAb	BL[a]	PY[b]	PP[c]	FP[d]
FX*n*#11	---	---[e]	---	---
FX*n*#18	---	---	---	---
FX*n*#111	---	---	---	---
FX*n*#218	0.20	---	---	---
FX*n*#222	---	---	---	---
FX*n*#226	---	---	---	---
FX*n*#233	2.10	---	---	---
FX*n*#313	5.60	---	0.40	---
FX*n*#356	---	---	0.30	---
FX*n*#362	---	---	---	---
FX*n*#368	7.60	---	---	---
FX*b*#21	---	---	0.30	---
FX*b*#113	---	---	0.40	---
FX*b*#115	---	---	---	---
FX*b*#119	566	---	5.10	0.80
FX*b*#120	132	---	1.20	0.20

[a] Boscalid. [b] Pyraclostrobin. [c] Penthiopyrad. [d] Fluopyram. [e] Cross-reactivity was lower than 0.1%.

Influence of pH and ionic strength

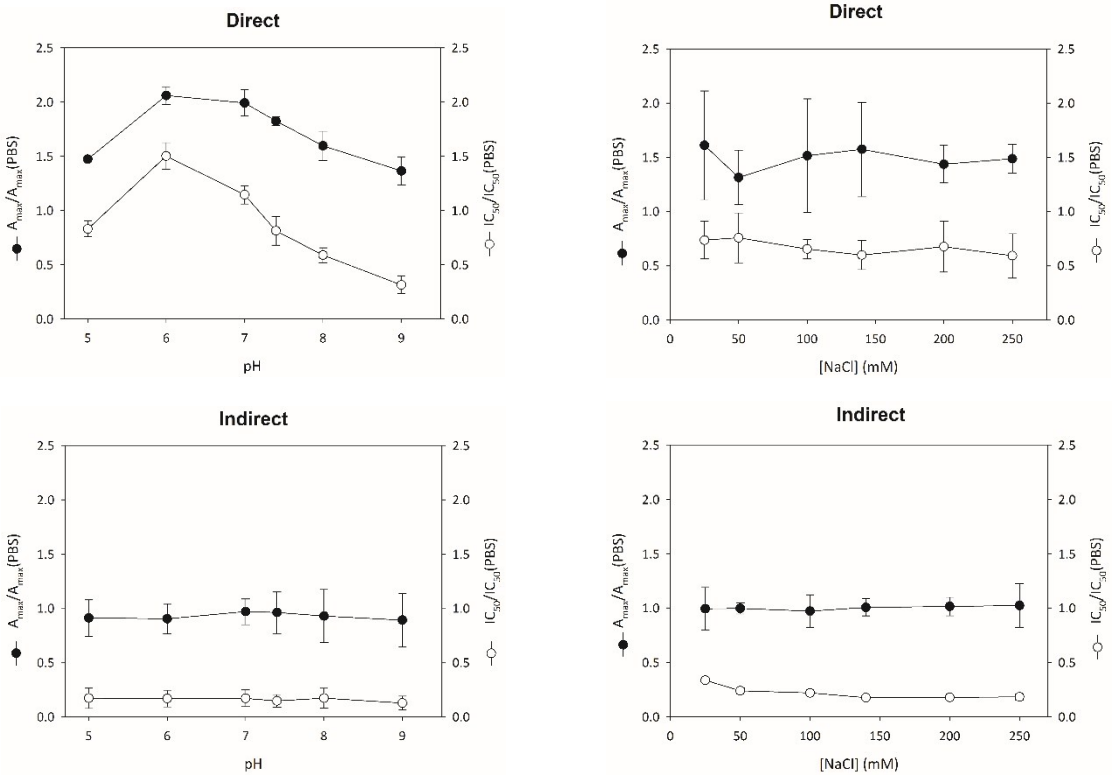


Figure S3. Influence of pH and NaCl concentration over the A_{max} and IC_{50} values of the studied immunoassays to fluxapyroxad.

Influence of organic solvents

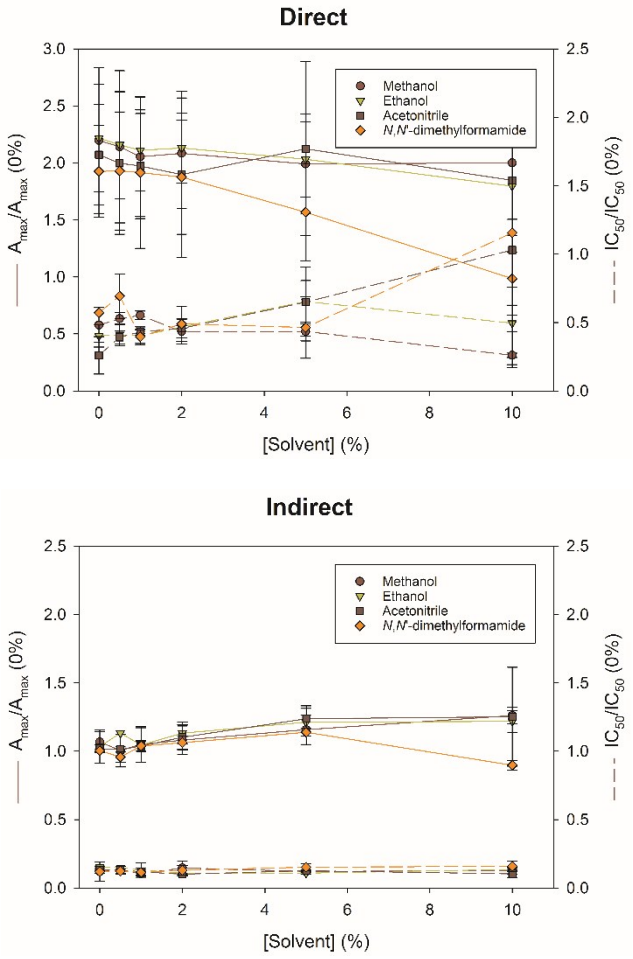


Figure S4. Influence of methanol, ethanol, acetonitrile, and *N*,*N*'-dimethylformamide over the A_{max} and IC_{50} values of the studied immunoassays to fluxapyroxad.

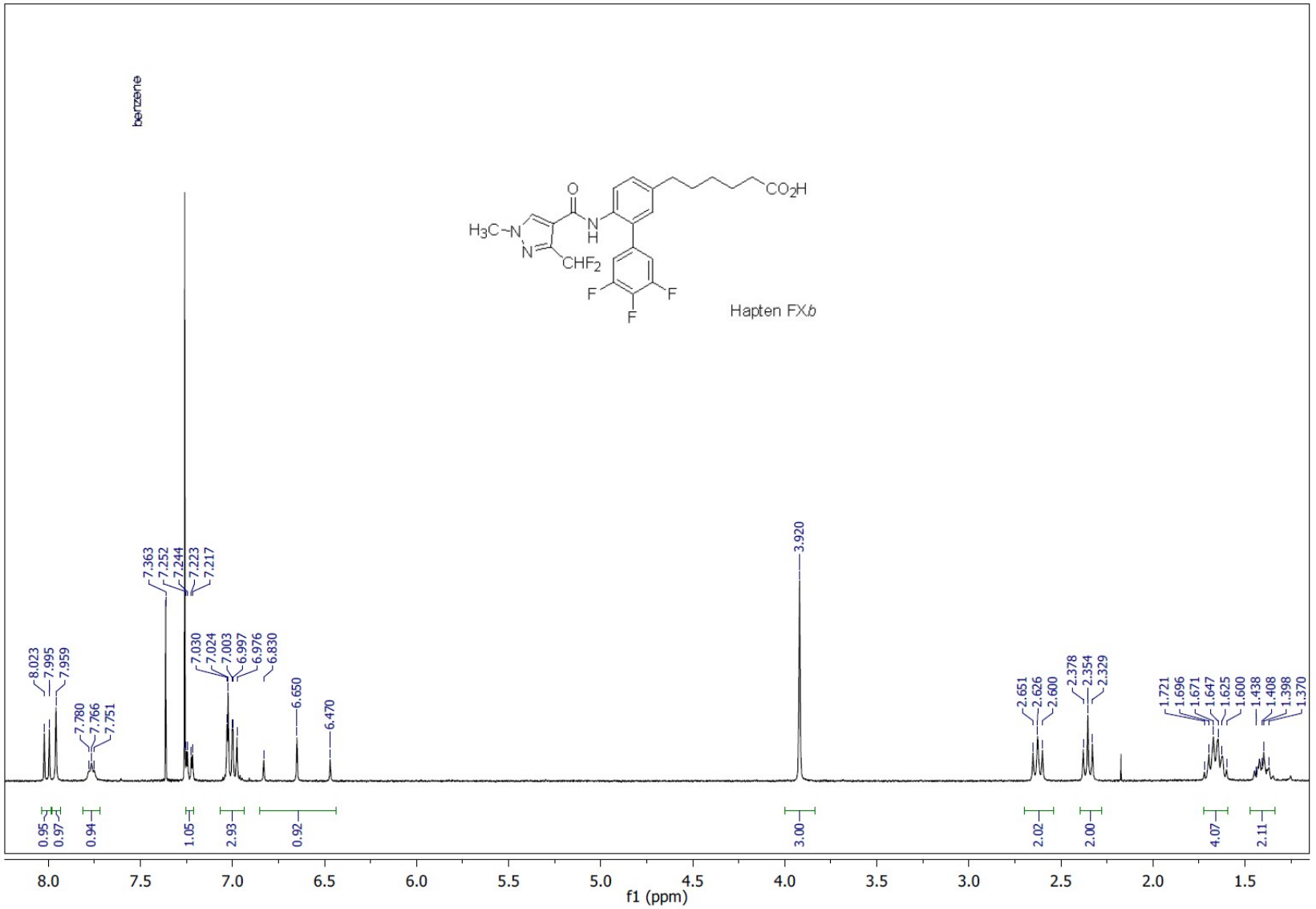
Analysis of in-field treated fruit samples by cELISA and UPLC–MS/MS.

Table S2. Analysis of fluxapyroxad-contaminated fruit extracts by the optimized indirect cELISA and a reference chromatographic method.

Sample code[a]	UPLC-MS/MS (ng/mL)	cELISA (ng/mL)	Sample code[b]	UPLC-MS/MS (ng/mL)	cELISA (ng/mL)
P-T1D1S1	1.24	8.86	GB-T1D1	189.58	156.01
P-T1D1S2	7.32	11.37	GB-T1D3	328.00	347.31
P-T1D3S1	0.28	7.13	GB-T2D1	226.38	207.75
P-T1D3S2	14.78	14.37	GB-T2D3	196.70	153.96
P-T1D5S1	0.10	7.59	GG-T1D1	393.75	438.28
P-T1D5S2	8.13	12.12	GG-T1D3	285.19	254.55
P-T1D7S1	2.35	8.74	GG-T2D1	389.18	425.25
P-T1D7S2	2.06	6.46	GG-T2D3	300.55	307.10
P-T2D1S1	61.96	37.25	GM-T1D1	128.20	85.07
P-T2D1S2	8.29	9.09	GM-T1D3	150.91	121.24
P-T2D3S1	6.07	12.03	GM-T2D1	611.73	713.91
P-T2D3S2	9.20	11.35	GM-T2D3	146.38	104.37
P-T2D5S1	1.99	8.83	GT-T1D1	307.74	321.55
P-T2D5S2	7.25	11.76	GT-T1D3	355.31	375.06
P-T2D7S1	9.84	13.45	GT-T2D1	138.91	103.68
P-T2D7S2	6.23	12.04	GT-T2D3	182.02	146.89

[a] P stands for plums, T for the type of treatment, D for day of sample collection, and S for the different samples that were collected. [b] GB stands for grapes var. Bobal, GG for grapes var. Garnacha, GM for grapes var. Macabeo, and GT for grapes var. Tempranillo.

^{1}H NMR spectrum of hapten FX*b* ($CDCl_3$, 300 MHz)



^{1}H NMR spectrum of hapten FX*h* ($CDCl_3$, 300 MHz)