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SUPPLEMENTARY INFORMATION

Rational design of a fluopyram hapten and preparation of bioconjugates and antibodies for immunoanalysis

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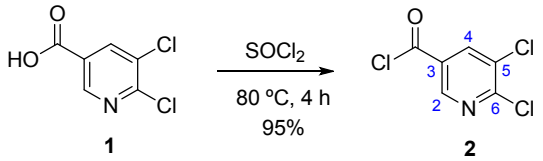
Materials and instruments

Pestanal grade fluopyram was purchased from Sigma/Aldrich (Madrid, Spain). Bovine serum albumin (BSA) fraction V was from Roche Applied Science (Mannheim, Germany). Ovalbumin (OVA), horseradish peroxidase (HRP) and adult bovine serum (ABS) were purchased from Sigma/Aldrich (Madrid, Spain). Sephadex G-25 HiTrap Desalting columns from GE Healthcare (Uppsala, Sweden) were utilized for protein–hapten conjugate purification. Goat anti-rabbit immunoglobulin polyclonal antibody conjugated to peroxidase was from BioRad (Madrid, Spain). Costar flat-bottom high-binding 96-well polystyrene ELISA plates were from Corning (Corning, NY, USA). UV–visible spectra and ELISA absorbances were read with a PowerWave HT from BioTek Instruments (Winooski, VT, USA). Microwells were washed with an ELx405 microplate washer also from BioTek Instruments. Solvents and reagents were purified by standard methods. All operations involving air-sensitive reagents were performed under an inert atmosphere of dry argon using syringe and cannula techniques, oven-dried glassware, and freshly distilled and dried solvents.

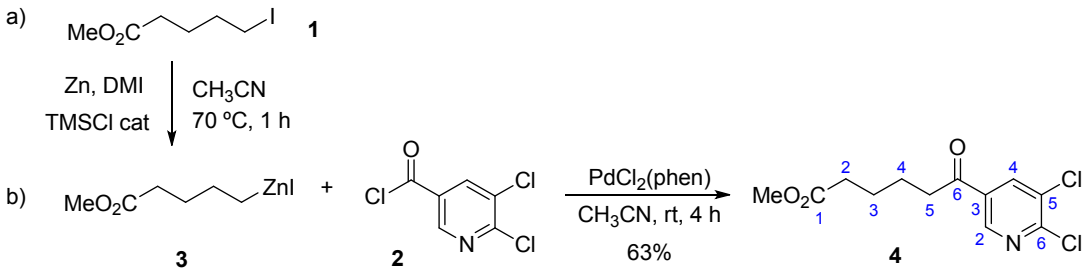
The progress of reactions was monitored by thin layer chromatography (TLC) performed on F_{254} silica gel plates. The plates were visualized at 254 nm by immersion with aqueous ceric ammonium molybdate and heating. Column chromatography refers to flash chromatography and was performed on Merck silica 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). 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). ^{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. Complete assignment of ^{1}H and ^{13}C chemical shifts of selected compound in the synthetic sequence was made on the basis of a combination of COSY and HSQC experiments. The abbreviation used for NMR data are as follows: s =singlet, d = doublet, dd = double doublet, t =

triplet, q = quadruplet, dt = double triplet, tt = triple triplet, br = broad, m = multiplet; Ph = phenyl ring, Py = pyridine ring. The molar extinction coefficient of haptens was determined in 100 mM phosphate buffer (PB), pH 7.4.

Synthesis of hapten and *N*-hydroxysuccinimidyl active ester



Preparation of 5,6-Dichloronicotinoyl chloride (**2**). A solution of 5,6-dichloronicotinic acid (**1**, 1.00 g, 5.21 mmol) and $SOCl_2$ (3.8 mL, 52.08 mmol) was stirred under reflux (80 °C) until TLC ($CHCl_3$/MeOH 95:5 as eluent) showed the consumption of all starting material (about 4h). The reaction mixture was cooled to room temperature, diluted with toluene and concentrated under vacuum to yield acyl chloride **2** (1.04 g, 95%) as a brownish solid that was used in the next step without further purification. Mp 45.6-47.0 °C (from toluene); IR (neat) ν_{max} (cm^{-1}) 3030 (m), 1750. (s), 1706 (m), 1562 (m), 1545 (m), 1416 (s), 1372 (s), 1198 (s), 1155 (s), 1043 (m), 929 (s); ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.97 (d, *J* = 2.0 Hz, 1 H, H_2 Py), 8.42 (d, *J* = 2.0 Hz, 1 H, H_4 Py); ^{13}C NMR (75 MHz, $CDCl_3$) δ 165.21 (CO), 155.85 (C_6 Py), 149.16 (C_2 Py), 140.23 (C_4 Py), 131.67 (C_3 Py), 129.30 (C_5 Py).



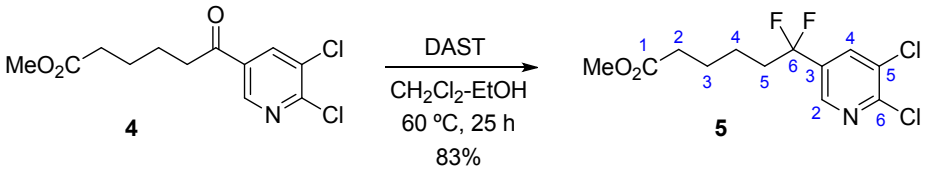
Preparation of methyl 6-(5,6-dichloropyridin-3-yl)-6-oxohexanoate (**4**).

(a) A suspension of activated dust zinc[1] (139.8 mg, 2.14 mmol) and 1,3-dimethyl-2-imidazolidinone (308 µL, 2.85 mmol) in anhydrous CH_3CN (3 mL) was sonicated in an ultrasonic bath for 5 minutes at room temperature under argon. The mixture was heated at 60 °C with stirring and two drops of trimethylchlorosilane were added. After 5 minutes, a solution of alkyl iodide **1** (690.1 mg, 2.85 mmol) in CH_3CN (3 mL) was added dropwise and the mixture stirred at 70

[1] W. L. F. *Armarego, D. D. Perrin. Purification of Laboratory Chemicals, 4th Ed.*, Butterworth-Heinemann, 2000, pag. 452.

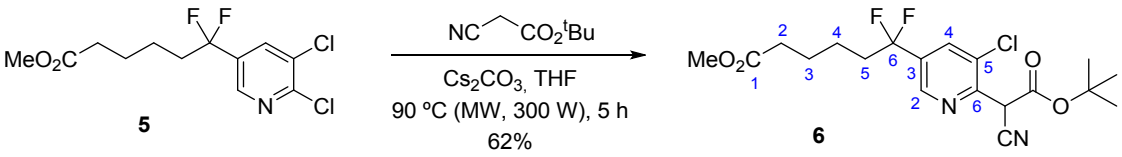
°C for 50-60 minutes to give a yellowish solution of 5-methoxy-5-oxopentyl)zinc(II) iodide (**3**).

(b) A solution of acyl chloride **2** (300 mg, 1.43 mmol) and $PdCl_2$(phen) (15.3 mg, 43 µmol) in anhydrous CH_3CN (3 mL) was dropwise added into the above prepared solution of organozinc **3** in CH_3CN, under argon atmosphere at room temperature. The resulting reaction mixture was stirred at room temperature for 4 h (reaction monitored by TLC using hexane-EtOAc 7:3 as eluent), then quenched by the addition of a saturated aqueous solution of NH_4Cl and extracted with EtOAc. The combined organic layers were washed successively with aqueous saturated NH_4Cl, 5% aqueous $NaHCO_3$ and brine, dried over anhydrous $MgSO_4$, filtered and concentrated under reduced pressure using a rotary evaporator. Purification of the obtained residue by chromatography, using hexane-EtOAc 9:1 as eluent, furnished the keto-ester **4** (262.6 mg, 63%) as a white solid. Mp 71.9-72.9 °C (from EtOAc); IR (neat) ν_{max} (cm^{-1}) 2953 (w), 1735 (s), 1684 (s), 1576 (m), 1431 (m), 1360 (s), 1282 (s), 1253 (m), 1153 (s), 1040 (s); ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.80 (d, *J* = 2.1 Hz, 1H, *H*$_2$ Py), 8.27 (d, *J* = 2.1 Hz, 1H, *H*$_4$ Py), 3.66 (s, 3H, CO_2CH_3), 2.98 (t, *J* = 6.9 Hz, 2H, *H*$_5$), 2.37 (t, *J* = 7.0 Hz, 2H, *H*$_2$), 1.84 – 1.65 (m, 4H, *H*$_3$ and *H*$_4$); ^{13}C NMR (75 MHz, $CDCl_3$) δ 196.39 (C$_6$), 173.78 (C$_1$), 153.26 (C$_6$ Py), 146.86 (C$_2$ Py), 137.92 (C$_4$ Py), 132.10 (C$_3$ Py), 131.46 (C$_5$ Py), 51.75 (CO_2CH_3), 38.82 (C$_5$), 33.84 (C$_2$), 24.41 (C$_4$), 23.17 (C$_3$); HRMS (TOF MS ES+) calcd for $C_{12}H_{14}Cl_2NO_3$ $[M+H]^+$ 290.0345, found 290.0342.

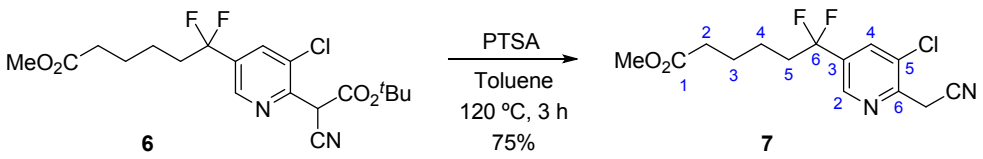


Preparation **of methyl 6-(5,6-dichloropyridin-3-yl)-6,6-difluorohexanoate** (**5**). Diethylaminosulfur trifluoride (DAST, 1.6 mL, 12.06 mmol) was added under argon to a solution of keto-ester **4** (350 mg, 1.21 mmol) in anhydrous CH_2Cl_2 (5.2 mL) and absolute EtOH (0.1 mL) contained in an ampoule cooled at -40 °C. The ampoule was sealed under vacuum and heated to 60 °C for 25 h. After this time, the ampoule was open and the content diluted with CH_2Cl_2 and water. The aqueous phase was extracted with CH_2Cl_2 and the combined organic layers washed with 5% aqueous $NaHCO_3$ solution and brine and dried over anhydrous $MgSO_4$. Chromatographic purification of the residue left after evaporation of the solvent at reduce pressure, using hexane-Et_2O 85:15 as eluent, afforded *gem*-difluorinated compound **5** (315.8 mg, 83%) as a yellowish oil. IR (neat) ν_{max} (cm^{-1}) 2954(w), 1735 (m), 1426 (w), 1372 (m), 1217 (m), 1155 (s), 998 (m), 838 (w),

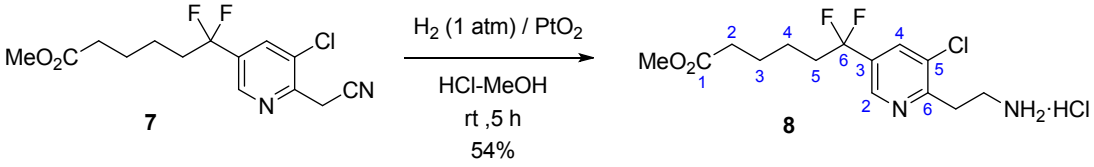
752 (s); ^{1}H NMR (300 MHz, $CDCl_3$) δ ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.40 (d, *J* = 2.2 Hz, 1H, H_2 Py), 7.85 (d, *J* = 2.2 Hz, 1H, H_4 Py), 3.67 (s, 3H, CO_2CH_3), 2.32 (t, *J* = 7.3 Hz, 2H, H_2), 2.24–2.04 (m, 2H, H_5), 1.69 (tt, *J* = 7.3, 7.3 Hz, 2H, H_3), 1.55–1.42 (m, 2H, H_4); ^{13}C NMR (75 MHz, $CDCl_3$) δ 173.62 (C_1), 150.90 (C_6 Py), 144.28 (t, $^3J_{C–F}$ = 6.6 Hz, C_2 Py), 135.83 (t, $^3J_{C–F}$ = 6.0 Hz, C_4 Py), 133.49 (t, $^2J_{C–F}$ = 28.2 Hz, C_3 Py), 130.92 (C_5 Py), 120.97 (t, $^1J_{C–F}$ = 243.9 Hz, C_6), 51.77 (CO_2CH_3), 38.68 (t, $^2J_{C–F}$ = 26.6 Hz, C_5), 33.70 (C_2), 24.43 (C_3), 21.90 (t, $^3J_{C–F}$ = 3.9 Hz, C_4); ^{19}F NMR (282 MHz, $CDCl_3$) δ -96.19 (CF_2); HRMS (TOF MS ES+) calcd for $C_{12}H_{14}Cl_2F_2NO_2$ $[M+H]^+$ 312.0364, found 312.0365.



Preparation* of *methyl 6-(6-(2-(tert-butoxy)-1-cyano-2-oxoethyl)-5-chloropyridin-3-yl)-6,6-difluorohexanoate (**6**). A mixture of chloropyridine **5** (100 mg, 0.32 mmol), *tert*-butyl 2-cyanoacetate (69 µL, 0.48 mmol) and Cs_2CO_3 (261 mg, 0.80 mmol) in anhydrous THF (1.5 mL) was placed in a microwave tube under a N_2 atmosphere. The mixture was then heated using a microwave oven at 60 °C (300 W) for 5h (reaction monitored by TLC using hexane-EtOAc 7:3 as eluent). The reaction mixture was poured into water and extracted with EtOAc, the combined organic layers were washed with brine, dried over anhydrous $MgSO_4$, filtered and concentrated at reduced pressure. The residue was purified by chromatography on silica gel, using hexane-EtOAc 9:1 as eluent, to provide compound **6** (82.7 mg, 62%) as a thick yellowish oil, whose ^{1}H NMR showed to be a 2:1 mixture of keto-enol tautomeric forms. IR (neat) ν_{max} (cm^{-1}) 2976 (w), 2962 (w), 2931 (w), 2201 (m), 1736 (s), 1636 (m), 1579 (m), 1369 (m), 1309 (m), 1244 (s), 1148 (s), 1005 (m); ^{1}H NMR (300 MHz, $CDCl_3$) (only signals of the major keto tautomer are given) δ 8.62 (d, *J* = 1.9 Hz, 1H, H_2 Py), 7.85 (d, *J* = 1.9 Hz, 1H, H_4 Py), 5.29 (s, 1H, $CH(CN)CO_2{}^t$Bu), 3.66 (s, 3H, CO_2CH_3), 2.33 (t, *J* = 7.3 Hz, 2H, H_2), 2.25–1.99 (m, 2H, H_5), 1.69 (tt, *J* = 7.3, 7.3 Hz, 2H, H_3), 1.50 (s, 9H, $CO_2C(CH_3)_3$), 1.56–1.43 (m, 2H, H_4); ^{13}C NMR (75 MHz, $CDCl_3$) (only signals of the major keto tautomer are given) δ 173.61 (C_1), 161.76 ($CO_2{}^t$Bu), 149.89 (C_6 Py), 144.83 (t, $^3J_{C–F}$ = 6.4 Hz, C_2 Py), 135.36 (t, $^2J_{C–F}$ = 28.1 Hz, C_3 Py), 134.88 (t, $^3J_{C–F}$ = 6.1 Hz, C_4 Py), 131.46 (C_5 Py), 120.95 (t, $^1J_{C–F}$ = 244.1 Hz, C_6), 113.89 (CN), 85.77 ($CO_2C(CH_3)_3$), 51.75 (CO_2CH_3), 44.96 ($CH(CN)CO_2{}^t$Bu), 38.67 (t, $^2J_{C–F}$ = 26.6 Hz, C_5), 33.70 (C_2), 27.85 ($CO_2C(CH_3)_3$), 24.44 (C_3), 21.86 (t, $^3J_{C–F}$ = 4.1 Hz, C_4); ^{19}F NMR (282 MHz, $CDCl_3$) δ -96.61 (CF_2); HRMS (TOF MS ES+) calcd for $C_{19}H_{24}ClF_2N_2O_4$ $[M+H]^+$ 417.1387, found 417.1385.

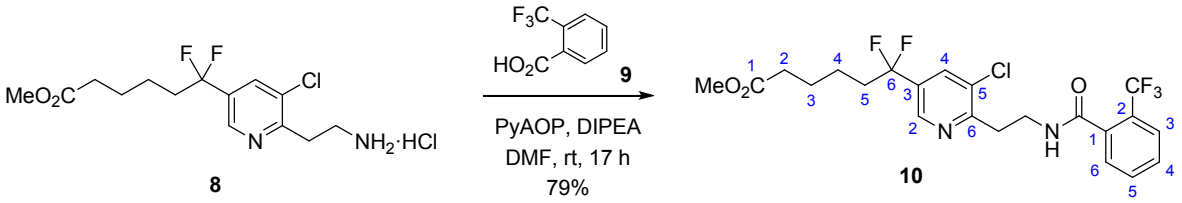


Preparation of methyl 6-(5-chloro-6-(cyanomethyl)pyridin-3-yl)-6,6-difluorohexanoate (**7**). A solution of *tert*-butyl ester **6** (150 mg, 0.36 mmol) and *p*-toluenesulfonic acid monohydrate (4.6 mg, 24 µmol) in anhydrous toluene (1.1 mL) was stirred under reflux under nitrogen for 3 h (reaction monitored by TLC using hexane-EtOAc 7:3 as eluent). The cooled reaction mixture was taken up in toluene and washed with 5% aqueous $NaHCO_3$ and brine and dried over anhydrous $MgSO_4$. Chromatographic purification of the residue left after evaporation of the solvent, using hexane-EtOAc 4:1 as eluent, afforded nitrile **7** (85.1 mg, 75%) as a yellowish solid. Mp 44.2-46.3 °C (from hexane-EtOAc); IR (neat) ν_{max} (cm^{-1}) 3005 (w), 2931 (w), 2874 (w), 2251 (w), 1725 (s), 1598 (w), 1389 (s), 1289. (m), 1167 (s), 1055 (m), 995 (s); ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.61 (d, *J* = 1.9 Hz, 1H, *H*$_2$ Py), 7.82 (d, *J* = 1.9 Hz, 1H, *H*$_4$ Py), 4.09 (s, 2H, C*H*$_2$CN), 3.66 (s, 3H, CO_2CH_3), 2.32 (t, *J* = 7.3 Hz, 2H, *H*$_2$), 2.24–2.06 (m, 2H, *H*$_5$), 1.68 (tt, *J* = 7.3, 7.3 Hz, 2H, *H*$_3$), 1.56–1.45 (m, 2H, *H*$_4$); ^{13}C NMR (126 MHz, $CDCl_3$) δ 173.61 (C$_1$), 149.69 (C$_6$ Py), 144.81 (t, $^3J_{C-F}$ = 6.4 Hz, *C*$_2$ Py), 134.67 (t, $^2J_{C-F}$ = 27.9 Hz, *C*$_3$ Py), 134.48 (t, $^3J_{C-F}$ = 6.0 Hz, *C*$_4$ Py), 130.98 (C$_5$ Py), 121.01 (t, $^1J_{C-F}$ = 243.8 Hz, *C*$_6$), 115.36 (CH_2CN), 51.75 (CO_2CH_3), 38.66 (t, $^2J_{C-F}$ = 26.6 Hz, *C*$_5$), 33.69 (C$_2$), 25.23 (C$_3$), 24.43 (CH_2CN), 21.90 (t, $^3J_{C-F}$ = 3.9 Hz, *C*$_4$); ^{19}F NMR (282 MHz, $CDCl_3$) δ -96.40 (C*F*$_2$); HRMS (TOF MS ES+) calcd for $C_{14}H_{16}ClF_2N_2O_2$ $[M+H]^+$ 317.0863, found 317.0859.



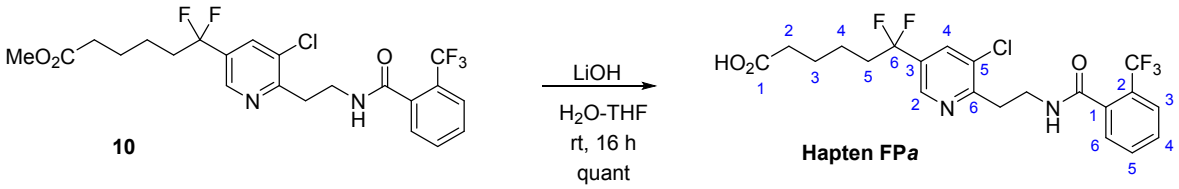
Methyl 6-(6-(2-aminoethyl)-5-chloropyridin-3-yl)-6,6-difluorohexanoate hydrochloride (**8**). A suspension of hydrochloride **7** (82.2 mg, 0.26 mmol) and PtO_2 (17.7 mg, 78 µmol) in a 0.35 M solution of HCl in MeOH (5.3 mL) was stirred at room temperature under hydrogen atmosphere (1 atm) for a period of 5 hours. After completion of the reaction as indicated by TLC (using $CHCl_3$-MeOH 4:1 as eluent), the reaction mixture was filtered through a pad of celite, using MeOH to wash. The solution was concentrated under vacuum and the residue purified by chromatography, using $CHCl_3$-MeOH 85:15 as eluent, to give amine hydrochloride **8** (54 mg, 58%) as an orange solid. Mp 99.5-100.7 °C (from $CHCl_3$-MeOH); IR (neat) ν_{max} (cm^{-1}) 3391 (m, br), 3070 (m), 2950 (m), 2915

(m), 1732 (s), 1594 (m), 1497 (m), 1380 (m), 1245 (m), 1165 (s), 985 (s); ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.53 (d, *J* = 1.6 Hz, 1H, H_2 Py), 7.75 (d, *J* = 1.9 Hz, 1H, H_4 Py), 7.25 (br s, 3H, $CH_2CH_2NH_3Cl$), 3.65 (s, 3H, CO_2CH_3), 3.53 (t, *J* = 5.6 Hz, 2H, CH_2CH_2N), 3.40 (t, *J* = 5.5 Hz, 2H, CH_2CH_2N), 2.31 (t, *J* = 7.3 Hz, 2H, H_2), 2.23–2.03 (m, 2H, H_5), 1.67 (t, *J* = 7.3 Hz, 2H, H_3), 1.55–1.42 (m, 2H, H_4); ^{13}C NMR (75 MHz, $CDCl_3$) δ 173.68 (C_1), 157.00 (C_6 Py), 143.93 (t, $^{3}J_{C–F}$ = 6.4 Hz, C_2 Py), 134.16 (t, $^{3}J_{C–F}$ = 6.0 Hz, C_4 Py), 133.21 (t, $^{2}J_{C–F}$ = 28.1 Hz, C_3 Py), 131.45 (C_5 Py), 121.18 (t, $^{1}J_{C–F}$ = 243.6 Hz, C_6), 51.73 (CO_2CH_3), 38.64 (t, $^{2}J_{C–F}$ = 26.7 Hz, C_5), 37.97 (CH_2CH_2N), 33.73 (C_2), 31.01 (CH_2CH_2N), 24.46 (C_3), 21.91 (t, $^{3}J_{C–F}$ = 3.9 Hz, C_4); ^{19}F NMR (282 MHz, $CDCl_3$) δ -96.25 (CF_2); HRMS (TOF MS ES+) calcd for $C_{14}H_{20}ClF_2N_2O_2$ $[M–Cl]^+$ 321.1176, found 321.1191.



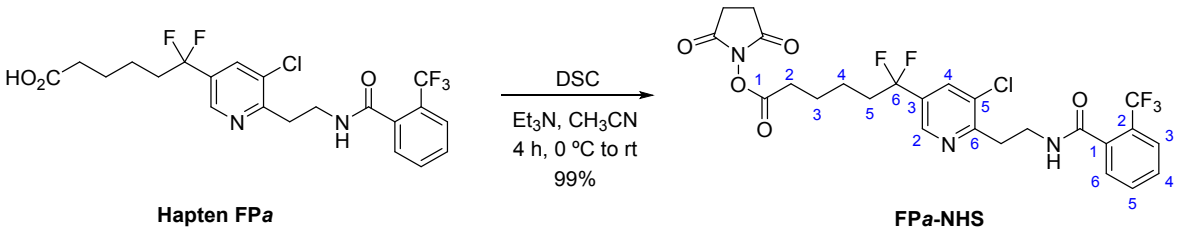
Methyl 6-(5-chloro-6-(2-(2-(trifluoromethyl)benzamido)ethyl)pyridin-3-yl)-6,6-difluoro-hexanoate (**10**). A solution of (7-azabenzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate (PyAOP, 189.7 mg, 0.36 mmol) in anhydrous DMF (1.5 mL) was dropwise added to a solution of amine hydrochloride **8** (100 mg, 0.28 mmol), 2-(trifluoromethyl)benzoic acid (**9**, 63.9 mg, 0.34 mmol) and *N,N*-diisopropylethylamine (DIPEA, 146 µL, 0.84 mmol) in DMF (1.5 mL) at room temperature under nitrogen. The resulting mixture was stirred for 17 h (reaction monitored by TLC using hexane-EtOAc 3:7 as eluent), then diluted with water and extracted with EtOAc. The combined organic layers were successively washed with 1.5% aqueous LiCl and brine, dried over anhydrous $MgSO_4$, filtered and the solvent removed under reduced pressure leaving a semi-solid. Chromatographic purification, using hexane-EtOAc 3:2 as eluent, gave amide **10** (109.2 mg, 79%) as a pale yellowish solid. Mp 44.5-47.2 °C (from Et_2O); IR (neat) ν_{max} (cm^{-1}) 3268 (m, ancha), 2945.96 (w), 2872.34 (w), 1725.30 (s), 1640.04 (s), 1543.16 (m), 1314.40 (s), 1169.27 (s), 1127 (s), 1059 (m), 985 (m); ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.47 (d, *J* = 2.0 Hz, 1H, H_2 Py), 7.75 (d, *J* = 2.0 Hz, 1H, H_4 Py), 7.65 (dd, *J* = 6.8, 2.2 Hz, 1H, H_6 Ph), 7.61–7.47 (m, 3H, H_3, H_4 and H_5 Ph), 6.74 (br t, *J* = 4.6 Hz, 1H, CONH), 3.99 (dt, *J* = 6.0 Hz, 2H, $NHCH_2CH_2$), 3.65 (s, 3H, CO_2CH_3), 3.26 (t, *J* = 5.9 Hz, 2H, $NHCH_2CH_2$), 2.30 (t, *J* = 7.3 Hz, 2H, H_2), 2.22–2.05 (m, 2H, H_5), 1.67 (dt, *J* = 7.4 Hz, 2H, H_3), 1.53–1.41 (m, 2H, H_4); ^{13}C NMR (75 MHz, $CDCl_3$) δ 173.68 (C_1), 167.78 (CONH), 158.41 (t, $^{5}J_{C–F}$ = 1.5 Hz, C_6 Py), 143.75 (t, $^{3}J_{C–F}$ = 6.5 Hz, C_2 Py), 136.30 (q, $^{3}J_{C–F}$ = 1.8 Hz, C_1 Ph), 133.82 (t, $^{3}J_{C–F}$ = 6.1 Hz, C_4 Py),

132.57 (t, $^{2}J_{C–F}$ = 28.0 Hz, *C*$_3$ Py), 132.16 (C$_5$ Ph), 131.79 (C$_5$ Py), 129.81 (C$_4$ Ph), 128.81 (C$_6$ Ph), 127.20 (q, $^{2}J_{C–F}$ = 32.3 Hz, *C*$_2$ Ph), 126.39 (q, $^{3}J_{C–F}$ = 5.0 Hz, *C*$_3$ Ph), 123.71 (q, $^{1}J_{C–F}$ = 273.6 Hz, *C*F$_3$), 121.34 (t, $^{1}J_{C–F}$ = 243.4 Hz, *C*$_6$), 51.73 (CO_2CH_3), 38.69 (t, $^{2}J_{C–F}$ = 26.9 Hz, *C*$_5$), 37.19 ($NHCH_2CH_2$), 33.87 ($NHCH_2CH_2$), 33.72 (C$_2$), 24.47 (C$_3$), 21.96 (t, $^{3}J_{C–F}$ = 5.7 Hz, *C*$_4$); ^{19}F NMR (282 MHz, $CDCl_3$) δ -59.47 (CF_3-Ph), -96.17 (C*F*$_2$); HRMS (TOF MS ES+) calcd for $C_{22}H_{23}ClF_5N_2O_3$ $[M+H]^+$ 493.1312, found 493.1318.



6-(5-Chloro-6-(2-(2-(trifluoromethyl)benzamido)ethyl)pyridin-3-yl)-6,6-difluorohexanoic acid (**hapten FP*a***). A 2.5 M aqueous solution of LiOH (1.1 mL, 2.74 mmol) was dropwise added to a well stirred solution of methyl ester **10** (82.2 mg, 0.17 mmol) in anhydrous THF (1.7 mL) and the mixture was stirred at room temperature for 16 h (reaction monitored by TLC using $CHCl_3$-MeOH 95:5 as eluent). Most of the solvent was distilled off under reduced pressure in a rotary evaporator, leaving a residue that was taken up in water and the resulting solution acidified to pH 2-3 with citric acid and extracted with EtOAc. The combined organic layers were washed with brine and dried over anhydrous $MgSO_4$, filtered, and the solvent removed under reduced pressure to give **hapten FP*a*** (79.9 mg, >99%) as a pale yellowish solid, which was nearly pure as shown by ^{1}H NMR spectroscopy. Mp 140.1-141.4 °C ($CHCl_3$); IR (neat) ν_{max} (cm^{-1}) 3289 (m, br), 2950 (w), 1710 (m), 1652 (m), 1541 (m), 1315 (s), 1174 (m), 1133 (m), 1065 (w), 1032 (w), 770 (m); ^{1}H NMR (300 MHz, CD_3OD) δ 8.59 (d, *J* = 1.8 Hz, 1H, *H*$_2$ Py), 7.96 (d, *J* = 1.9 Hz, 1H, *H*$_4$ Py), 7.72 (dd, *J* = 7.4, 0.8 Hz, 1H, *H*$_3$ Ph), 7.70–7.63 (m, 1H, *H*$_5$ Ph), 7.63–7.57 (m, 1H, *H*$_4$ Ph), 7.48 (dd, *J* = 7.5, 0.6 Hz, 1H, *H*$_6$ Ph), 3.84 (t, *J* = 6.9 Hz, 2H, $CONHCH_2CH_2$), 3.30 (t, *J* = 6.9 Hz, 2H, $CONHCH_2CH_2$), 2.27 (t, *J* = 7.2 Hz, 2H, *H*$_2$), 2.33–2.14 (m, 2H, *H*$_5$), 1.64 (tt, *J* = 7.2, 7.2 Hz, 2H, *H*$_3$), 1.53–1.42 (m, 2H, *H*$_4$); ^{13}C NMR (75 MHz, CD_3OD) δ 177.21 (C$_1$), 170.68 ($CONHCH_2CH_2$), 159.17 (C$_6$ Py), 145.11 (t, $^{3}J_{C–F}$ = 6.6 Hz, *C*$_2$ Py), 137.21 (q, $^{3}J_{C–F}$ = 2.4 Hz, *C*$_1$ Ph), 135.27 (t, $^{3}J_{C–F}$ = 6.1 Hz, *C*$_4$ Py), 134.19 (t, $^{2}J_{C–F}$ = 28.1 Hz, *C*$_3$ Py), 133.30 (C$_5$ Ph), 132.79 (C$_5$ Py), 130.97 (C$_4$ Ph), 129.56 (C$_6$ Ph), 128.23 (q, $^{2}J_{C–F}$ = 32.0 Hz, *C*$_2$ Ph), 127.40 (q, $^{3}J_{C–F}$ = 5.0 Hz, *C*$_3$ Ph), 125.13 (q, $^{1}J_{C–F}$ = 272.9 Hz, CF_3-Ph), 122.87 (t, $^{1}J_{C–F}$ = 242.3 Hz, *C*$_6$), 39.10 (t, $^{2}J_{C–F}$ = 26.7 Hz, *C*$_5$), 38.85 ($CONHCH_2CH_2$), 35.29 (C$_2$), 34.54 ($CONHCH_2CH_2$), 25.50 (C$_3$), 23.00 (t, $^{3}J_{C–F}$ = 4.0 Hz, *C*$_4$); ^{19}F NMR (282 MHz, CD_3OD) δ -60.94 (CF_3-Ph), -96.99 (C*F*$_2$); HRMS (TOF

MS ES+) calcd for $C_{21}H_{21}ClF_5N_2O_3$ $[M+H]^+$ 479.1155, found 479.1157.



Hapten FP*a*

FP*a*-NHS

2,5-Dioxopyrrolidin-1-yl 6-(5-chloro-6-(2-(2-(trifluoromethyl)benzamido)ethyl)pyridin-3-yl)-6,6-difluorohexanoate (**FP*a*-NHS ester**). Anhydrous Et_3N (14 µL, 96 µmol) was added to a stirred solution of hapten FP*a* (12.2 mg, 26 µmol) and *N,N'*-succinimidyl carbonate (8.4 mg, 34 µmol) in anhydrous CH_3CN (500 µL) at 0 °C under nitrogen. The reaction mixture was stirred at room temperature for 4 h and then diluted with water and extracted with EtOAc. The organic layers were combined and dried over anhydrous $MgSO_4$ and then concentrated under vacuum, to give nearly pure *N*-hydroxysuccinimidyl ester of hapten FP*a* (**FP*a*-NHs ester**, 14.6 mg, 99%) as a colorless oil. IR (neat) ν_{max} (cm^{-1}) 3295 (d, br), 2951 (w), 2923 (w), 1810 (m), 1785 (m), 1736 (s), 1657 (m), 1527 (m), 1314 (s), 1204 (m), 1129 (m), 1062 (s), 1034 (m), 754 (m); ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.48 (d, *J* = 2.0 Hz, 1H, H_2 Py), 7.76 (d, *J* = 2.0 Hz, 1H, H_4 Py), 7.68–7.63 (m, 1H, H_3 Ph), 7.61–7.46 (m, 3H, H_4, H_5 and H_6 Ph), 6.75 (br t, *J* = 5.3 Hz, 1H, CON*H*CH_2CH_2), 3.98 (dt, *J* = 6.0, 6.0 Hz, 2H, CONHC$H_2$$CH_2$), 3.27 (t, *J* = 5.9 Hz, 2H, CONHCH_2CH_2), 2.81 (s, 4H, CO(CH_2)$_2$CO, 2.60 (t, *J* = 7.2 Hz, 2H, H_2), 2.25–2.07 (m, 2H, H_5), 1.80 (dt, *J* = 7.5, 7.5 Hz, 2H, H_3), 1.63–1.53 (m, 2H, H_4); ^{13}C NMR (75 MHz, $CDCl_3$) δ 169.15 (*CO*(CH_2)$_2$*CO*), 168.24 (C_1), 167.78 (CONH), 158.46 (C_6 Py), 143.75 (t, $^3J_{C-F}$ = 6.4 Hz, C_2 Py), 136.31 (q, $^3J_{C-F}$ = 2.2 Hz, C_1 Ph), 133.84 (t, $^3J_{C-F}$ = 6.1 Hz, C_4 Py), 132.38 (t, $^2J_{C-F}$ = 27.7 Hz, C_3 Py), 132.15 (C_5 Ph), 131.82 (C_5 Py), 129.81 (C_4 Ph), 128.79 (C_6 Ph), 127.21 (q, $^2J_{C-F}$ = 31.3 Hz, C_2 Ph), 126.43 (q, $^3J_{C-F}$ = 4.9 Hz, C_3 Ph), 123.71 (q, $^1J_{C-F}$ = 274.3 Hz, CF_3-Ph), 121.27 (t, $^1J_{C-F}$ = 243.4 Hz, C_6), 38.49 (t, $^2J_{C-F}$ = 27.0 Hz, C_5), 37.21 (NHCH_2CH_2), 33.88 (NHC$H_2$$CH_2$), 30.79 ($C_2$), 25.69 (s, CO($CH_2$)$_2$CO), 24.21 ($C_3$), 21.67 (t, $^3J_{C-F}$ = 4.0 Hz, C_4); ^{19}F NMR (282 MHz, $CDCl_3$) δ -59.45 (CF_3-Ph), -96.13 (CF_2); HRMS (TOF MS ES+) calcd for $C_{25}H_{24}ClF_5N_3O_5$ $[M+H]^+$ 576.1319, found 576.1316.

Conjugation to proteins

Preparation of immunogenic and assay conjugates

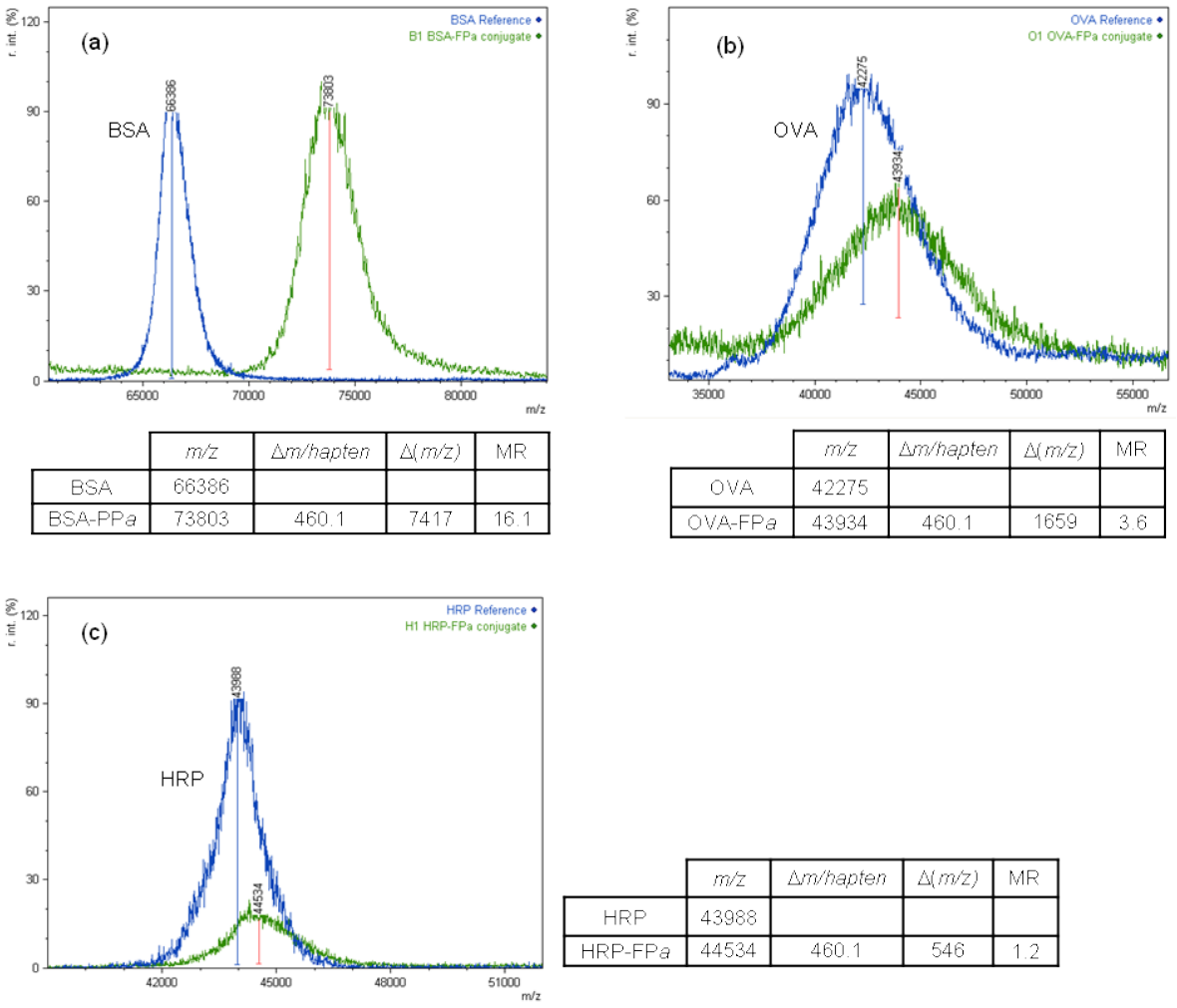
The succinimidyl ester of hapten FP*a* was dissolved in *N*,*N*-dimethylformamide and drop wise added to a protein solution in 50 mM carbonate buffer, pH 9.6, under gentle stirring, never exceeding a 10% (v/v) DMF final concentration. As carrier proteins BSA, OVA, and horseradish peroxidasa (HRP) for immunogen, coating antigen, and enzyme tracer preparation were respectively employed. The coupling reaction was carried out during 2 h at room temperature in amber glass vials with initial molar hapten-to-protein ratios of 24, 8, and 11 for BSA, OVA, and HRP, respectively. Next, bioconjugates were purified by size-exclusion chromatography in Sephadex G-25 using 100 mM phosphate buffer, pH 7.4, as eluent. The immunizing BSA conjugate was filter sterilized and stored frozen at −20 °C.

MALDI mass spectrometry analysis of immunogenic and assay conjugates

Sample preparation. 100 µL of each of the above protein conjugate solutions (0.5-1 mg/mL) were dialyzed against milliQ water and then freeze-dried and lyophilized. The samples were dissolved in H_2O MilliQ to theoretical final concentration 1 µg/µL. One microliter 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 5800 MALDI TOFTOF (ABSciex) 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 the TOFTOF 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 prepared bioconjugates showed final molar ratios (MR) of 16.1, 3.6, and 1.2 for BSA-FP*a*, OVA-FP*a*, and HRP-FP*a*, respectively. (Fig. S1).



	m/z	*Δm/hapten*	*Δ(m/z)*	MR
BSA	66386			
BSA-PP*a*	73803	460.1	7417	16.1

	m/z	*Δm/hapten*	*Δ(m/z)*	MR
OVA	42275			
OVA-FP*a*	43934	460.1	1659	3.6

	m/z	*Δm/hapten*	*Δ(m/z)*	MR
HRP	43988			
HRP-FP*a*	44534	460.1	546	1.2

Figure S1. MALDI-TOF-MS spectra of proteins (blue) and the corresponding conjugates with hapten FP*a* (green): (a) BSA and BSA-hapten PP*a*; (b) OVA and OVA-hapten FP*a*; (c) HRP and HRP-hapten FP*a.*

Antibody generation

Two antisera were generated from two 2-kg female New Zealand white rabbits, which had been immunized with 21-day intervals by subcutaneous injection of 0.3 mg of BSA–FP*a* conjugate in 1 mL of a 1:1 emulsion between sterile 100 mM phosphate, pH 7.4, and Freund's adjuvant (complete for the first dose and incomplete for subsequent boosts). Ten days after the third injection blood samples were collected from the ear vein, and ten day after the fourth injection, rabbits were exsanguinated. Blood samples were allowed to coagulate overnight at 4 °C, and sera were separated by centrifugation. Finally, antibodies were precipitated with 1 volume of saturated ammonium sulphate solution. Salting out was performed twice, and precipitates were stored at 4 °C.

Conjugate-coated indirect cELISA

Coating was carried out in sealed plates by incubation overnight at room temperature with 100 µL per well of OVA–FP*a* conjugate solution in coating buffer. After washing as described, the competitive step was done with 50 µL per well of fluopyram standard solution in PBS and 50 µL per well of antibody dilution in PBST, and incubation during 1 h at room temperature. Then, plates were washed again, and 100 µL per well of secondary enzyme-labelled antibody (diluted 1/10000 in PBST carrying 10% (v/v) ABS) was added and incubated 1 h at room temperature. Signal was obtained and enzymatic activity was stopped as mentioned for direct assays.

Antibody-coated direct cELISA

Coating for direct cELISAs was performed by overnight incubation at 4 °C in sealed plates bearing 100 µL per well of antibody dilution in cold 50 mM carbonate buffer, pH 9.6 (coating buffer). Then, microwells were rinsed four times with a 150 mM NaCl and 0.05% (v/v) Tween 20 solution. The competitive step was carried out during 1 h at room temperature by mixing 50 µL per well of fluopyram standard solution in PBS (10 mM phosphate, pH 7.4 containing 140 mM NaCl) and 50 µL per well of HRP-FP*a* tracer solution in PBST (PBS with 0.05% (v/v) Tween 20). After washing as described before, signal was generated by adding 100 µL per well of freshly prepared *o*-phenylendiamine (2 mg/mL) solution containing 0.012% (v/v) H_2O_2 in 25 mM citrate and 62 mM phosphate buffer, pH 5.4, and incubation during 10 min at room temperature. Finally, 100 µL per well of 1 M H_2SO_4 was added.

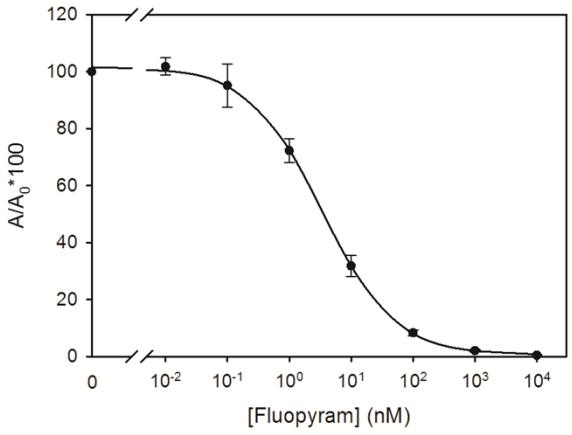
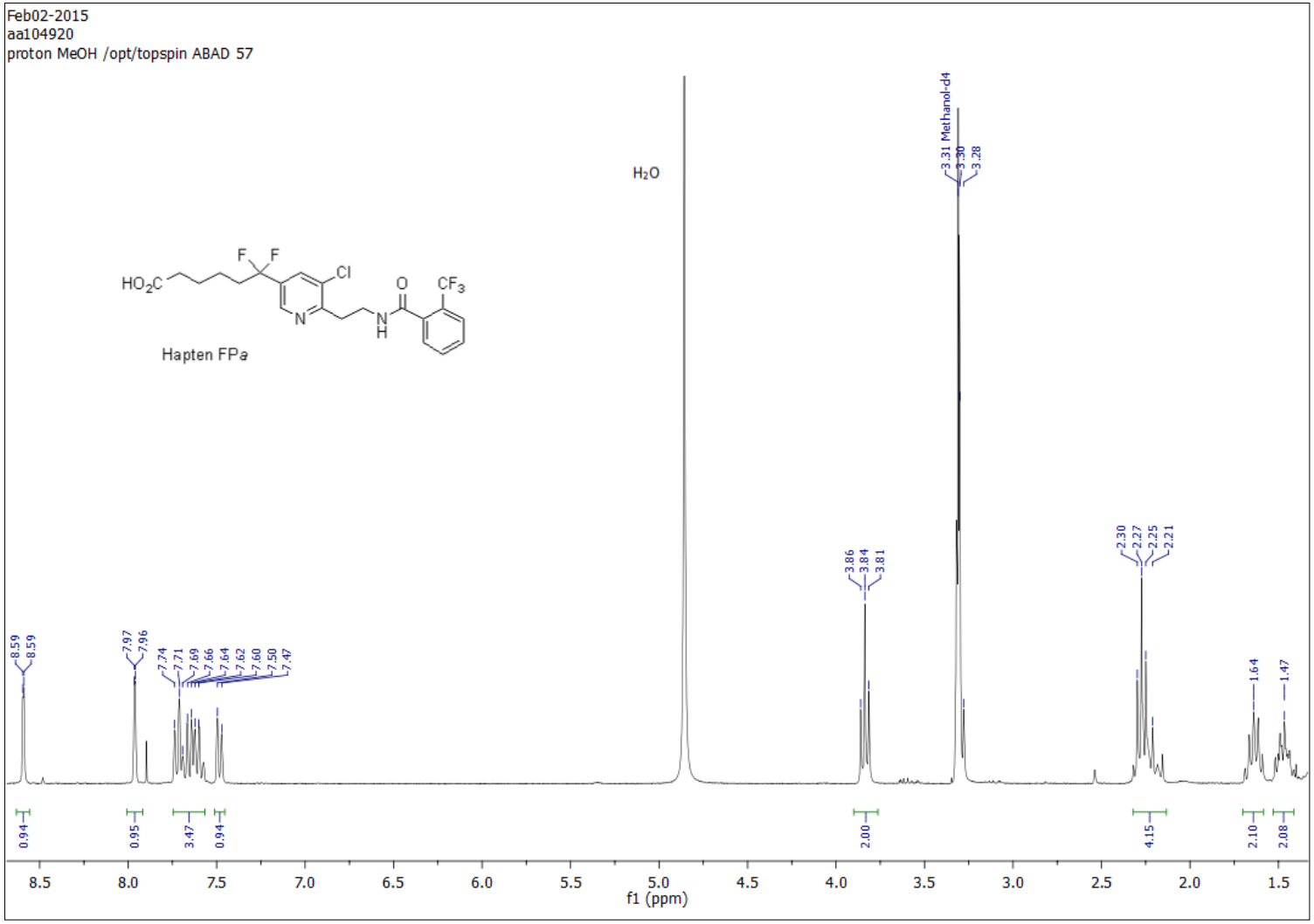


Fig. S2. Fluopyram inhibition curve by direct competitive ELISA using antiserum FP*a*#2.

Copies of 1H NMR spectra

^{1}H NMR spectra of hapten FP*a* (300 MHz, CD_3OD)



^{1}H NMR spectra of FP*a*–NHS ester (300 MHz, $CDCl_3$)