ELECTRONIC SUPPLEMENTARY INFORMATION

Haptens, bioconjugates, and antibodies for penthiopyrad immunosensing

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General methods and instruments

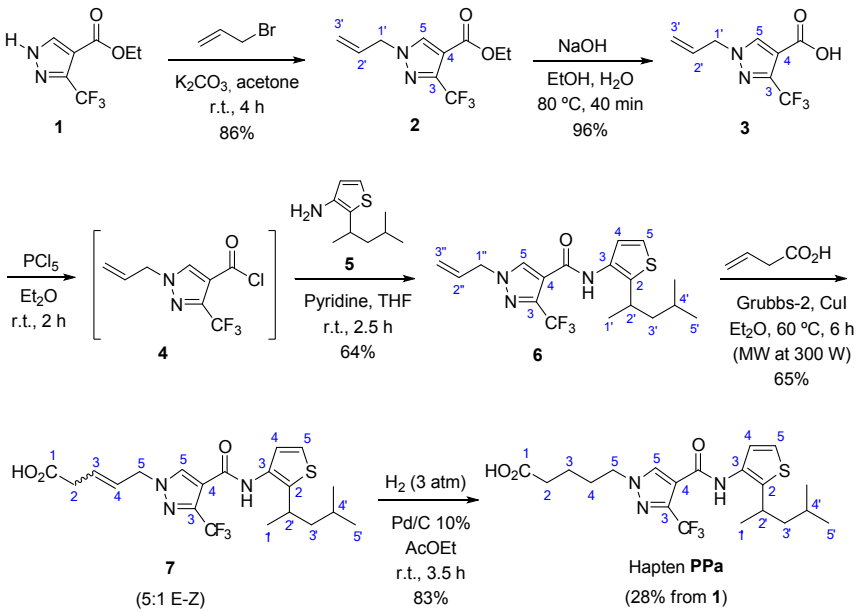
Pestanal grade penthiopyrad ((RS)-*N*-[2-(1,3-dimethylbutyl)-3-thienyl]-1-methyl-3-(trifluoromethyl)pyrazole-4-carboxamide, CAS registry number 131341-86-1, Mw 359.41) was purchased from DuPont (Nambsheim, France). Bovine serum albumin (BSA) fraction V was from Roche Applied Science (Mannheim, Germany). 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 MeOH-d_4); ^{13}C NMR: 77.00 ppm for $CDCl_3$ and 49.00 ppm for MeOH-d_4). ^{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 molar extinction coefficient of haptens was determined in 100 mM phosphate buffer (PB), pH 7.4.

Synthesis of hapten PP*a*

The synthesis of hapten PP*a* started with the initial preparation of *N*-allyl pyrazole **2**, via alkylation of commercially available **1** with allyl bromide (Scheme 1A in the manuscript). The allylation reaction was highly regioselective in favor of the *N*-1 alkylated product (40:1 ratio of regioisomeric *N*-1/*N*-2 alkylated products). Completion of the synthesis of the bicyclic penthiopyrad skeleton was achieved by hydrolysis of the ethyl ester moiety followed by amidation reaction of the corresponding acyl chloride with 3-aminothiophene **5**, readily prepared from methyl 3-aminothiophene-2-carboxylate by a modification of the literature method.[1,2] Further elaboration of the five carbon-length carboxylated side chain that completed the synthesis of hapten PP*a* involved a microwave assisted cross-metathesis reaction of **6** with 3-butenoic acid to give **7**, followed by double-bond hydrogenation under mild conditions. Thus, the synthesis of hapten PP*a* was accomplished from **1** via six steps in a 28% overall yield.

[1] H. Katsuta, S. Ishii, K. Tomiya and K. Kodaka, *Eur. Pat.*, EP1036793 (A2), 2000.
[2] D. Ura, H. Katsuta, T. Kitashima and K. Sato, WO2004009581 (A1), 2004.



Scheme S1

Ethyl 1-allyl-3-(trifluoromethyl)-1*H*-pyrazole-4-carboxylate (2). K_2CO_3 (498.0 mg, 3.60 mmol) and allyl bromide (311 µL, 3.60 mmol) were added to a suspension of ethyl 1-methyl-3-(trifluoromethyl)-1*H*-pyrazole-4-carboxylate (**1**, 500.0 mg, 2.40 mmol) in anhydrous acetone (5.4 mL) under N_2 atmosphere. The resulting mixture was stirred at room temperature for 4 h (reaction monitored by TLC, hexane/EtOAc 7:3). After this time, the mixture was diluted in water and then extracted with EtOAc. The combined organic layers were washed with brine and dried over anhydrous $MgSO_4$, then filtered and concentrated under vacuum. The crude product was purified by flash column chromatography (silica gel, hexane/EtOAc 9:1), to give allyl derivative **2** as a white solid (513.2 mg, 86%).

Physical and spectroscopic data: Mp 54.4–55.0 °C (hexane) [lit.,[3] a yellow solid]; IR (KBr) ν_{max} (cm^{-1}) 3419.8w, 3158.9m, 2995.1m, 2936.8m, 1907.6w, 1728.1s, 1541.s, 1307.7s, 1058.7s, 860.9s, 774.7s; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.99 (d, *J* = 0.9 Hz, 1H, H_5 Pz), 6.01 (ddt, *J* = 16.4, 10.2, 6.2 Hz, 1H, H_2 allyl), 5.38 (ddt, *J* = 10.2, 2.1, 1.1 Hz, 1H, $H_{3'}$ allyl), 5.32 (ddt, *J* = 16.4, 2.1, 1.2 Hz, 1H, $H'_{3'}$ allyl), 4.78 (d, *J* = 6.2 Hz, 2H, $H_{1'}$ allyl), 4.30 (q, *J* = 7.1 Hz, 2H, OCH_2CH_3), 1.33 (t, *J* = 7.1 Hz, 3H, OCH_2CH_3); ^{13}C NMR (75 MHz, $CDCl_3$) δ 160.93 (CO_2Et), 141.72 (q, $^2J_{C\text{-}F}$ = 38.4 Hz, C_3 Pz), 135.29 (C_5 Pz), 130.89 ($C_{2'}$ allyl), 120.97 ($C_{3'}$ allyl), 120.50 (q, $^1J_{C\text{-}F}$ = 269.6 Hz, CF_3), 113.62 (C_4 Pz), 61.04 (OCH_2CH_3), 55.69 ($C_{1'}$ allyl), 14.17 (OCH_2CH_3); ^{19}F NMR (282 MHz, $CDCl_3$) δ −62.49 (s); HRMS (ES) calculated for $C_{10}H_{12}N_2O_2F_3$ $[M+H]^+$ 249.0845 , found 249.0845.

1-Allyl-3-(trifluoromethyl)-1*H*-pyrazole-4-carboxylic acid (3). A mixture of ethyl ester **2** (450.0 mg, 1.81 mmol), aqueous NaOH 1 M solution (3.6 mL, 3.62 mmol) and EtOH (5 mL) was

[3] Novel herbicides. Black, Janice; Boehmer, Jutta Elisabeth; Chrystal, Ewan James Turner; Kozakiewicz, Anthony Marian; Plant, Andrew. PCT Int. Appl. (2007), WO 2007071900 A1, 2007-06-28.

refluxed (85 °C) for 30 minutes (reaction monitored by TLC, Hexane/EtOAc 7:3). After this time, the mixture was cooled in an ice-water bath, acidified to pH 2 with concentrated HCl and then extracted with EtOAc. The combined organic layers were washed with brine and dried over anhydrous $MgSO_4$, then filtered and concentrated under vacuum to afford acid **3** as a white solid which was used in the next step without further purification (399.3 mg, 96%).

Physical and spectroscopic data: Mp 139.6–140.3 °C (hexane/$CHCl_3$); IR (KBr) ν_{max} (cm^{-1}) 3157.5s, 2987.8s, 2600.1s, 1701.3s, 1541.4s, 1316.9s, 1150.9s, 1052.8s, 874.6s, 742.6s; ^{1}H NMR (300 MHz, $CDCl_3$) δ 10.52 (br s, 1H, O*H*), 8.06 (d, *J* = 0.6 Hz, 1H, H_4 Pz), 6.02 (ddt, *J* = 16.4, 10.2, 6.3 Hz, 1H, $H_{2'}$ allyl), 5.41 (ddd, *J* = 10.2, 2.0, 1.1 Hz, 1H, $H_{3'}$ allyl), 5.35 (ddd, *J* = 17.0, 2.3, 1.4 Hz, 1H, $H'_{3'}$ allyl), 4.81 (d, *J* = 6.2 Hz, 2H, $H_{2'}$ allyl); ^{13}C NMR (75 MHz, $CDCl_3$) δ 166.44 (CO_2H), 142.38 (q, $^2J_{C\text{-}F}$ = 38.7 Hz, C_3 Pz), 136.32 (C_5 Pz), 130.63 ($C_{2'}$ allyl), 121.29 ($C_{3'}$ allyl), 120.30 (q, $^1J_{C\text{-}F}$ = 269.8 Hz, CF_3), 112.58 (C_4 Pz), 55.78 ($C_{1'}$ allyl); ^{19}F NMR (282 MHz, $CDCl_3$) δ −62.65 (s); HRMS (ES) calculated for $C_8H_8N_2O_2F_3$ $[M+H]^+$ 221.0532, found 221.0523.

1-Allyl-*N*-(2-(4-methylpentan-2-yl)thiophen-3-yl)-3-(trifluoromethyl)-1*H*-pyrazole-4-carboxamide (6). A suspension of acid **3** (120.1 mg, 0.55 mmol) and PCl_5 (125.0 mg, 0.60 mmol) in anhydrous Et_2O (1.81 mL) was stirred at room temperature for 2 h under N_2 atmosphere until a transparent solution was formed (reaction monitored by TLC, hexane-EtOAc 7:3). At that point, the solvent was evaporated under vacuum, and the residue containing the intermediate acyl chloride **4** was dissolved in anhydrous THF (0.91 mL) and treated with anhydrous pyridine (88 µL, 1.09 mmol) and aminothiophene **5** (100 mg, 0.55 mmol), prepared as described below. The resulting mixture was stirred at room temperature for 2.5 h (reaction monitored by TLC, hexane/EtOAc 7:3), then diluted in EtOAc and washed sequentially with aqueous solutions of HCl (1 M), $NaHCO_3$ (5%) and brine. The organic layer was dried over anhydrous $MgSO_4$, then filtered and concentrated under vacuum. The residue obtained was purified by flash column chromatography (silica gel, hexane/EtOAc 4:1) to give amide **6** as a white solid (135.5 mg, 64%).

Physical and spectroscopic data: Mp. 130.6–131.0 °C (benzene); IR (KBr) ν_{max} (cm^{-1}) 3226.2m, 3195.1m, 3125. 7w, 2956.2m, 1640.6s, 1566.4s, 1491.3m, 1211.8m, 1141.6s, 1058.2s, 895.8w; ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.09 (s, 1H, H_5 Pz), 7.55 (s, 1H, N*H*), 7.42 (d, *J* = 5.4 Hz, 1H, H_4 Th), 7.12 (d, *J* = 5.4 Hz, 1H, H_5 Th), 6.02 (ddt, *J* = 16.6, 10.2, 6.3 Hz, 1H, $H_{2''}$ allyl), 5.40 (d, *J* = 10.2 Hz, 1H, $H_{3''}$ allyl), 5.35 (d, *J* = 16.6 Hz, 1H, $H_{3''}$ allyl), 4.79 (d, *J* = 6.2 Hz, 2H, $H_{1''}$ allyl), 3.08 (sext, *J* = 7.0 Hz, 1H, $H_{2'}$), 1.68–1.38 (m, 3H, $H_{3'}$ and $H_{4'}$), 1.25 (d, *J* = 6.8 Hz, 3H, $H_{1'}$), 0.86 (d, *J* = 5.8 Hz, 6H, $H_{5'}$ and *Me-4'*); ^{13}C NMR (75 MHz, $CDCl_3$) δ 158.32 (*C*ONH), 139.57 (C_3 Th), 137.77 (q, $^2J_{C\text{-}F}$ = 36.9 Hz, C_3 Pz), 135.61 (C_5 Pz), 130.81 ($C_{2''}$ allyl), 129.74 (C_2 Th), 124.34 (C_4 Th), 121.31 (q, $^1J_{C\text{-}F}$ = 269.2 Hz, CF_3), 121.24 (C_5 Th), 121.14 ($C_{3''}$ allyl), 117.48 (C_4 Pz), 55.80 ($C_{1''}$

allyl), 48.13 (*C*$_{3'}$), 30.33 (*C*$_{2'}$), 25.77 (*C*$_{4'}$), 23.17 (*C*$_{1'}$), 22.62 and 22.54 (*C*$_{5'}$ and *Me-4'*); ^{19}F NMR (282 MHz, $CDCl_3$) δ −59.69 (s); HRMS (ES) calculated for $C_{18}H_{23}F_3N_3OS$ $[M+H]^+$ 386.1508, found 386.1508.

5-(4-(2-(4-Methylpentan-2-yl)thiophen-3-ylcarbamoyl)-3-(trifluoromethyl)-1*H*-pyrazol-1-yl)pent-3-enoic acid (7). But-3-enoic acid (33 µL, 0.39 mmol) was added to a suspension of **6** (50.0 mg, 0.13 mmol), 2nd Generation Grubbs Catalyst[4] (6.6 mg, 7.78 µmol) and CuI (2.0 mg, 10.38 µmol) in anhydrous Et_2O (1.7 mL) under N_2. The resulting mixture was stirred at 60 °C for 6 h under microwave irradiation (300 W). After this time, the solvent was evaporated under vacuum and the residue obtained was purified by flash column chromatography (silica gel). A CH_2Cl_2/Et_2O 95:5 mixture was initially used as eluent to recover 7.6 mg (15%) of starting material **6**; then, the eluent was changed to 4:1 of the same mixture to give the unsaturated acid **7** (37.4 mg, 65%, as a 5:1 mixture of *E*-*Z* isomers) as a colourless oil.

Spectroscopic data: IR (KBr) ν_{max} (cm^{-1}) 3261.9s (broad), 3126.2w, 2958.9s, 1716.1s, 1651.8s, 1488.4s, 1306.2s, 1143.4s, 1057.1s, 973.6m, 833.6m, 715.4m; ^{1}H NMR data of the major (*E*) isomer (300 MHz, $CDCl_3$), δ 9.31 (br s, 1H, O*H*), 8.16 (s, 1H, *H*$_5$ Pz), 7.57 (s, 1H, N*H*), 7.40 (d, *J* = 5.4 Hz, 1H, *H*$_4$ Th), 7.12 (d, *J* = 5.4 Hz, 1H, *H*$_5$ Th), 5.97 (dt, *J* = 15.3, 6.5 Hz, 1H, *H*$_3$), 5.84 (dt, *J* = 15.8, 6.3 Hz, 1H, *H*$_4$), 4.80 (d, *J* = 6.2 Hz, 2H, *H*$_5$), 3.20 (d, *J* = 6.3 Hz, 2H, *H*$_2$), 3.08 (sext, *J* = 7.0 Hz, 1H, *H*$_{2'}$), 1.66–1.39 (m, 3H, *H*$_{3'}$ and *H*$_{4'}$), 1.24 (d, *J* = 6.8 Hz, 3H, *H*$_{1'}$), 0.86 (d, *J* = 6.3 Hz, 6H, *H*$_{5'}$ and *Me-4'*); ^{13}C NMR data of the major (*E*) isomer (75 MHz, $CDCl_3$) δ 175.47 (*C*$_1$), 158.53 (*C*ONH), 139.85 (*C*$_3$ Th), 137.76 (q, $^2J_{C\text{-}F}$ = 43.1 Hz, *C*$_3$ Pz), 135.75 (*C*$_5$ Pz), 129.59 (*C*$_2$ Th), 129.09 (*C*$_4$ Th), 126.85 (*C*$_4$), 124.34 (*C*$_3$), 121.29 (*C*$_5$ Th), 121.27 (q, $^1J_{C\text{-}F}$ = 270.4 Hz, *C*F_3), 117.36 (*C*$_4$ Pz), 54.86 (*C*$_5$), 48.12 (*C*$_{3'}$), 37.08 (*C*$_2$), 30.34 (*C*$_{2'}$), 25.78 (*C*$_{4'}$), 23.18 (*C*$_{1'}$), 22.62 and 22.53 (*C*$_{5'}$ and *Me-4'*); ^{19}F NMR (282 MHz, $CDCl_3$) δ −59.67 (s); HRMS (ES) calculated for $C_{20}H_{25}F_3N_3O_3S$ $[M+H]^+$ 444.1563, found 444.1553.

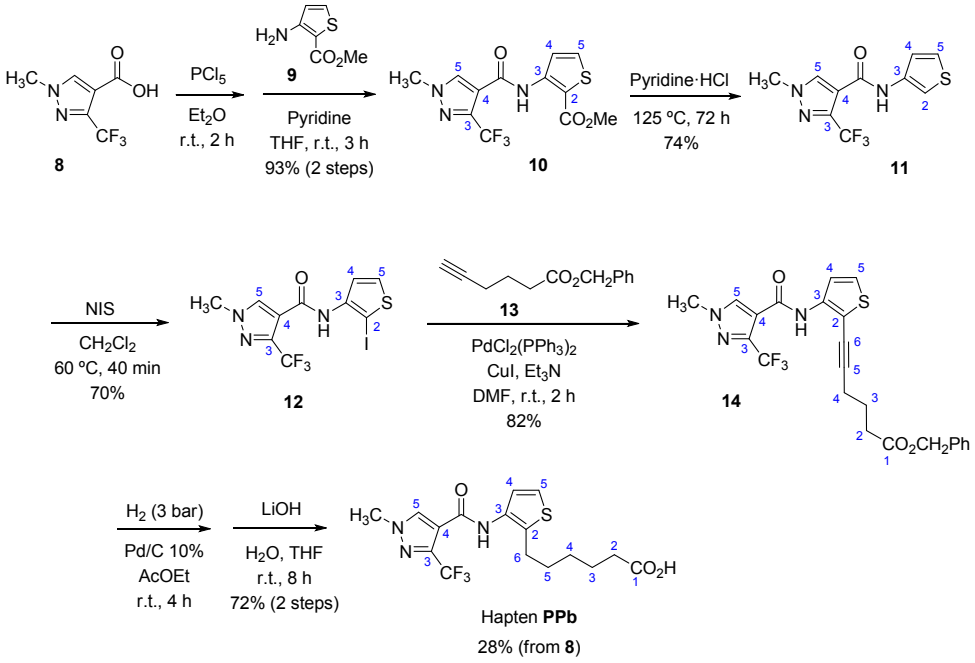
5-(4-(2-(4-Methylpentan-2-yl)thiophen-3-ylcarbamoyl)-3-(trifluoromethyl)-1*H*-pyrazol-1-yl)pentanoic acid (Hapten PP*a*). A Büchi 'Tiny Clave' reactor equipped with a magnetic stirring bar was charged with **7** (80.0 mg, 0.18 mmol), 10% Pd/C (68.0 mg) and EtOAc (4 mL). The charged reactor was purged five times with 3–5 bar of H_2 and then pressurized to 3 bar. The reaction mixtures were stirred at room temperature for 3.5 h. Next, the reactor was depressurised and the black suspension was filtered through a pad of celite with EtOAc and the collected filtrate was concentrated in vacuum. The obtained residue was purified by flash column chromatography (silica gel, $CHCl_3$/MeOH 98:2) to give hapten **PP*a*** as a white solid (66.9 mg, 83%).

[4] (1,3-*Bis*(2,4,6-trimethylphenyl)-2-imidazolidinylidene)dichloro(phenylmethylene)

Physical and spectroscopic data: Mp 148.3–149.4 °C (benzene); IR (KBr) ν_{max} (cm^{-1}) 3031.3m, 2962.1m, 2261.0w, 2086.43w, 1697.3s, 1408.4m, 1285.0m, 1195.9m, 928.w, 688.8w; ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.42 (br s, 1H, O*H*), 8.15 (s, 1H, H_5 Pz), 7.62 (s, 1H, N*H*), 7.39 (d, *J* = 5.6 Hz, 1H, H_4 Th), 7.12 (d, *J* = 5.4 Hz, 1H, H_5 Th), 4.19 (t, *J* = 7.0 Hz, 2H, H_5), 3.09 (sext, *J* = 6.8 Hz, 1H, $H_{2'}$), 2.40 (t, *J* = 7.1 Hz, 2H, H_2), 2.05–1.88 (m, 2H, H_4), 1.73–1.61 (m, 2H, H_3), 1.61–1.39 (m, 3H, $H_{3'}$ and $H_{4'}$), 1.24 (d, *J* = 6.8 Hz, 3H, $H_{1'}$), 0.86 (d, *J* = 6.1 Hz, 6H, $H_{5'}$ and *Me-4'*); ^{13}C NMR (75 MHz, $CDCl_3$) δ 178.21 (*C*O_2H), 158.68 (*C*ONH), 139.95 (C_3 Th), 137.75 (q, $^{2}J_{C\text{-}F}$ = 37.0 Hz, C_3 Pz), 135.98 (C_5 Pz), 129.56 (C_2 Th), 124.36 (C_4 Th), 121.28 (q, $^{1}J_{C\text{-}F}$ = 269.5 Hz, *C*F_3), 121.23 (C_5 Th), 116.86 (C_4 Pz), 52.94 (C_5), 48.09 ($C_{3'}$), 33.23 (C_2), 30.30 ($C_{2'}$), 29.22 (C_4), 25.73 ($C_{4'}$), 23.13 ($C_{1'}$), 22.58 and 22.49 ($C_{5'}$ and *Me-4'*), 21.56 (C_3); ^{19}F NMR (282 MHz, MeOH-d_4) δ −64.08 (s); HRMS (ES) calculated for $C_{20}H_{27}F_3N_3O_3S$ $[M+H]^+$ 446.1720, found 446.1707. UV (PB) ε (280 nm) 2.67 mM^{-1} cm^{-1}.

Synthesis of hapten PP*b*

The synthesis of hapten PP*b* involved the initial preparation of the already known biheteroaromatic system **11** from pyrazole-carboxylic acid **8** (Scheme 1B in the manuscript).[1] In this case, the incorporation of the carboxylated spacer-arm was based on a Sonogashira cross-coupling reaction. Thus, iodination of the *C*-2 position of the thiophene ring of **11**, followed by palladium-catalyzed cross-coupling reaction of the resulting iodide with benzyl hex-5-ynoate (**13**) afforded the acetylenic derivative **14**, which after hydrogenation of the triple bond and hydrolysis of the ester moiety, led to hapten PP*b*. Overall, the synthesis of this hapten proceeded in seven steps with a global yield of ca. 30%.



Scheme S2

Methyl 3-(1-methyl-3-(trifluoromethyl)-1*H*-pyrazole-4-carboxamido)thiophene-2-carboxylate (10). A suspension of carboxylic acid **8** (1 g, 4.63 mmol) and PCl_5 (1.06 g, 5.09 mmol) in anhydrous Et_2O (16 mL) was stirred at room temperature under anhydrous conditions for 2 h. The clear solution obtained after this time was evaporated to dryness under vacuum to afford the crude acyl chloride intermediate which was dissolved in anhydrous THF (7.7 mL) and treated, under an atmosphere of argon, with anhydrous pyridine (749 µL, 9.26 mmol) and methyl 3-aminotiophene-2-carboxylate (**9**, 727.8 mg, 4.63 mmol). The reaction mixture was stirred at room temperature for 3 h, diluted with EtOAc and washed successively with water, dilute hydrochloric acid, dilute aqueous $NaHCO_3$, and finally brine, dried over anhydrous. $MgSO_4$, filtered, and concentrated under reduced pressure. The reaction product was purified by flash column chromatography (silica gel, CH_2Cl_2/Et_2O 95:5), to give amide **10** as a white solid (1.2756 g, 93%).

Physical and spectroscopic data: Mp 144.0–144.8 °C (MeOH) [lit.,[5] colourless crystals]; IR (KBr) ν_{max} (cm^{-1}) 3303.2w, 3128.4w, 2956.3w, 1679.0s, 1447.6m, 1301.1s, 1173.4s, 1060.2s, 782.7s, 651.2m; ^{1}H NMR (300 MHz, $CDCl_3$) δ 10.68 (s, 1H, N*H*), 8.19 (d, *J* = 5.5 Hz, 1H, H_5 Th), 7.95 (s, 1H, H_5 Pz), 7.51 (d, *J* = 5.0 Hz, 1H, H_4 Th), 4.02 (s, 3H, NCH_3), 3.91 (s, 3H, CO_2CH_3); ^{13}C NMR (75 MHz, $CDCl_3$) δ 165.22 (*C*ONH), 157.55 (CO_2CH_3), 144.69 (C_3 Th), 141.23 (q, $^2J_{C\text{-}F}$ = 38.4 Hz, C_3 Pz), 133.21 (C_4 Th), 132.08 (C_5 Pz), 122.57 (C_5 Th), 120.64 (q, $^1J_{C\text{-}F}$ = 269.6 Hz, CF_3), 116.94 (C_4 Pz), 110.70 (C_2 Th), 52.22 (CO_2CH_3), 40.07 (NCH_3); ^{19}F NMR (282 MHz, $CDCl_3$) δ −61.64 (s); HRMS (ES) calculated for $C_{12}H_{11}F_3N_3O_3S$ $[M+H]^+$ 334.0468, found 334.0468.

1-Methyl-*N*-(thiophen-3-yl)-3-(trifluoromethyl)-1*H*-pyrazole-4-carboxamide (11). A mixture of methyl ester **10** (1.28 g, 3.83 mmol) and pyridine hydrochloride (1.33 g, 11.48 mmol) in anhydrous pyridine (11.3 mL) was stirred at 130 °C under nitrogen for 72 h (reaction monitored by TLC, CH_2Cl_2/Et_2O 7:3). The mixture was cooled to room temperature, poured into ice-water, acidified to pH 1 with concentrated HCl and extracted with EtOAc. The combined organic layers were washed with brine, dried over anhydrous $MgSO_4$, filtered and concentrated under vacuum to give a solid residue that was purified by flash column chromatography (silica gel, CH_2Cl_2/Et_2O 95:5) affording the decarboxylated thiophene **11** as a pale brown solid (777.6 mg, 74%).

Physical and spectroscopic data: Mp 156.6–157.2 °C (benzene/EtOAc) [lit.,[3] brown crystals]; IR (KBr) $\nu_{máx}$ (cm^{-1}) 3566.8w, 3342.7s, 3126.9m, 2942.4w, 1647.9s, 1538.0s, 1499.3s, 1308.6s, 836.4m, 774.0s; ^{1}H NMR (300 MHz, MeOH-d_4) δ 8.22 (d, *J* = 0.5 Hz, 1 H, H_5 Pz), 7.60 (dd, *J* = 3.2, 1.3 Hz, 1 H, H_2 Th), 7.35 (dd, *J* = 5.2, 3.2 Hz, 1 H, H_4 Th), 7.16 (dd, *J* = 5.2, 1.3 Hz, 1 H, H_5 Th), 3.99 (s, 3 H, CO_2CH_3); ^{13}C NMR (75 MHz, MeOH-d_4) δ 160.51 (*C*ONH), 141.80 (q, $^2J_{C\text{-}F}$ = 38.0 Hz, C_3 Pz), 137.37 (C_3 Th), 134.61 (C_5 Pz), 125.34 (C_4 Th), 122.55 (C_5 Th), 122.23 (q, $^1J_{C\text{-}F}$ = 268.5 Hz, CF_3), 117.69 (C_4 Pz), 111.44 (C_2 Th), 39.88 (NCH_3); ^{19}F NMR (282 MHz, MeOH-d_4) δ −62.90 (s); HRMS (ES) calculated for $C_{10}H_9F_3N_3OS$ $[M+H]^+$ 276.0413, found 276.0419.

***N*-(2-Iodothiophen-3-yl)-1-methyl-3-(trifluoromethyl)-1*H*-pyrazole-4-carboxamide (12).** A suspension of thiophene **11** (250.0 mg, 0.91 mmol) and *N*-iodosuccinimide (206.1 mg, 0.91 mmol) in anhydrous CH_2Cl_2 (36.7 mL) was stirred in the darkness at 65 °C under N_2 atmosphere for 40 minutes (reaction monitored by TLC, $CHCl_3$/Et_2O 9:1). After this time, the solvent was evaporated under vacuum, and the residue was purified by flash column chromatography (silica gel, $CHCl_3$) to give iodo-thiophene **12** as a pale brown solid (253.6 mg, 70%).

[5] Hiroyuki Katsuta, Seiichi Ishii, Kanji Tomiya, Kenji Kodaka. A process for preparing 2-alkyl-3-aminothiophene derivative and 3-aminothiophene derivative. Eur. Pat. Appl. (2000), EP 1036793 A2, 2000-09-20.

Physical and spectroscopic data: Mp 163.2–164.1 °C (benzene/hexane); IR (KBr) ν_{max} (cm^{-1}) 3396.8m, 3117.4w, 3085.8m, 2953.6w, 1675.2s, 1568.9s, 1474.9m, 1378.6m, 1296.4m, 1064.6s; ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.03 (s, 1H, *H*$_5$ Pz), 7.80 (s, 1H, N*H*), 7.72 (d, *J* = 5.8 Hz, 1H, *H*$_5$ Th), 7.51 (d, *J* = 5.7 Hz, 1H, *H*$_4$ Th), 4.00 (s, 3H, C*H*$_3$); ^{13}C NMR (75 MHz, $CDCl_3$) δ 157.72 (*C*ONH), 139.61 (*C*$_3$ Th), 138.57 (q, $^2J_{C\text{-}F}$ = 37.4 Hz, *C*$_3$ Pz), 136.11 (*C*$_5$ Pz), 130.57 (*C*$_4$ Th), 123.04 (*C*$_5$ Th), 121.01 (q, $^1J_{C\text{-}F}$ = 269.5 Hz, *C*F_3), 116.98 (*C*$_4$ Pz), 62.46 (*C*$_3$ Th), 40.07 (N*C*H_3); ^{19}F NMR (282 MHz, $CDCl_3$) δ −59.52 (s); HRMS (ES) calculated for $C_{10}H_8F_3IN_3OS$ $[M+H]^+$ 401.9379, found 401.9368.

Benzyl 6-(3-(1-methyl-3-(trifluoromethyl)-1*H*-pyrazole-4-carboxamido)thiophen-2-yl)hex-5-ynoate (14). Triethylamine (360 µL, 2.58 mmol) was added under N_2 atmosphere to a stirred suspension of iodide **12** (180.0 mg, 0.45 mmol), $PdCl_2(PPh_3)_2$ (9.5 mg, 13.46 µmol), CuI (6.0 mg, 31.42 µmol) and benzyl hex-5-ynoate[6] (**13,** 136.2 mg, 0.67 mmol) in anhydrous DMF (360 µL). The resulting mixture was stirred at room temperature for 2 h (reaction monitored by TLC, CH_2Cl_2/Et_2O 9:1). After this time, the solution was diluted in water and then extracted with EtOAc. The combined organic layers were washed with a 1.5% aqueous solution of LiCl and brine, dried over anhydrous $MgSO_4$, filtered and concentrated under vacuum. The crude product was purified by flash column chromatography (silica gel, CH_2Cl_2/Et_2O 95:5) to give compound **14** as a brownish oil (175.0 mg, 82%).

Spectroscopic data: IR (neat) $\nu_{máx}$ (cm^{-1}) 3418.7w, 2946.0w, 1731.9m, 1683.0m, 1673.9m, 1588.5s, 1425.0s, 1295.7m, 1172.1s, 1139.7s, 1058.8, 881.3w, 755.5m; ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.30 (s, 1H, N*H*), 8.09 (d, *J* = 0.6 Hz, 1H, *H*$_5$ Pz), 7.89 (d, *J* = 5.5 Hz, 1H, *H*$_5$ Th), 7.39–7.27 (m, 5H, CH_2*Ph*), 7.14 (dd, *J* = 5.5, 0.3 Hz, 1H, *H*$_4$ Th), 5.10 (s, 2H, C*H*$_2$Ph), 3.97 (s, 3H, C*H*$_3$), 2.59 (t, *J* = 6.8 Hz, 2H, *H*$_2$), 2.54 (t, *J* = 7.0 Hz, 2H, *H*$_4$), 1.98 (tt, *J* = 7.0 Hz, 1H, *H*$_3$); ^{13}C NMR (75 MHz, $CDCl_3$) δ 173.19 (*C*O_2), 157.19 (*C*ONH), 139.76 (*C*$_3$ Th), 138.58 (q, $^2J_{C\text{-}F}$ = 37.6 Hz, *C*$_3$ Pz), 136.14 (*C*$_5$ Pz), 135.94 (*C*$_1$ Ph), 128.70 (*C*$_3$ and *C*$_5$ Ph), 128.39 (*C*$_4$ Ph), 128.25 (*C*$_2$ and *C*$_6$ Ph), 125.22 (*C*$_4$ Th), 121.65 (*C*$_5$ Th), 121.07 (q, $^1J_{C\text{-}F}$ = 269.7 Hz, *C*F_3), 117.11 (d, *J* = 0.8 Hz, *C*$_4$ Pz), 107.21 (*C*$_2$ Th), 99.42 (*C*$_6$), 71.58 (*C*$_5$), 66.52 (s, *C*H_2Ph), 40.00 (N*C*H_3), 33.34 (*C*$_2$), 23.69 (*C*$_3$), 19.46 (*C*$_4$); ^{19}F NMR (282 MHz, $CDCl_3$) δ −60.00 (s); HRMS (ES) calculated for $C_{23}H_{21}N_3O_3F_3S$ $[M+H]^+$ 476.1250, found 476.1247.

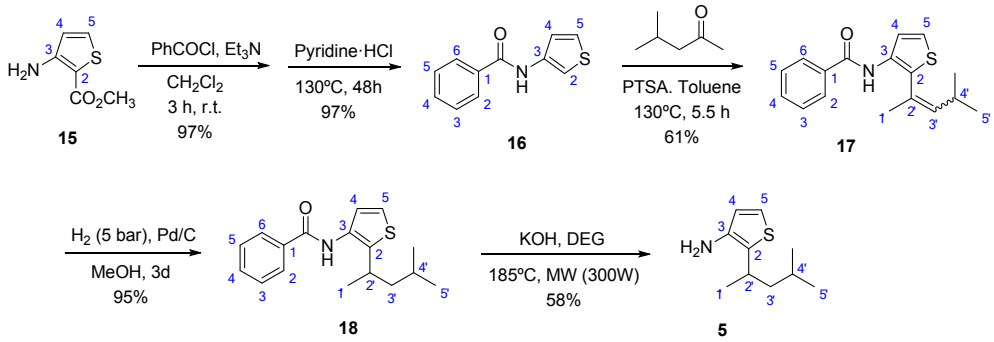
6-(3-(1-Methyl-3-(trifluoromethyl)-1*H*-pyrazole-4-carboxamido)thiophen-2-yl)hexanoic acid (Hapten PP*b*). A suspension of **14** (175.0 mg, 0.37 mmol) and 10% Pd/C (85.0 mg) in

[6] David C. M. Chan, Hongning Fu, Ronald A. Forsch, Sherry F. Queener. Design, Synthesis, and Antifolate Activity of New Analogues of Piritrexim and Other Diaminopyrimidine Dihydrofolate Reductase Inhibitors with ω-Carboxyalkoxy or ω-Carboxy-1-alkynyl Substitution in the Side Chain. *J. Med. Chem*., **2005**, *48*, 4420-4431.

anhydrous EtOAc (4.4 mL) was hydrogenated according to the procedure described above for the hydrogenation of compound **7**. In this case, the mixture was reacted for 4 h under a pressure of hydrogen of 3 bar. The residue obtained after filtration and evaporation of the solvent under vacuum was dissolved in THF (3.5 mL) and a 2.5 M aqueous solution of LiOH (2 mL, 4.97 mmol)and the resulting mixture was stirred at room temperature for 8 h (reaction monitored by TLC, $CHCl_3$/MeOH 95:5). After this time, the THF was evaporated under vacuum and the remaining aqueous solution was acidified with 1 M hydrochloric acid to pH and extracted with EtOAc. The combined organic layers were washed with brine and dried over anhydrous $MgSO_4$, then filtered and concentrated under vacuum. The residue obtained was purified by flash column chromatography (silica gel, $CHCl_3$/MeOH 99:1 to 95:5), to give hapten **PP*b*** as a white solid (103.7 mg, 72%).

Physical and spectroscopic data: Mp 106.3–107.7 °C (benzene/EtOAc); IR (KBr) ν_{max} (cm^{-1}) 3262.5s, 2943.6m, 1719.5s, 1650.6s, 1574.6m, 1487. 7m, 1211.4s, 1122.5s, 896.0w; ^{1}H NMR (300 MHz, $CDCl_3$) δ 10.85 (br s, 1H, O*H*), 8.03 (s, 1H, H_5 Pz), 7.70 (s, 1H, N*H*), 7.34 (d, *J* = 4.9 Hz, 1H, H_4 Th), 7.06 (d, *J* = 5.4 Hz, 1H, H_5 Th), 3.92 (s, 3H, NCH_3), 2.68 (t, *J* = 7.6 Hz, 2H, H_6), 2.31 (t, *J* = 7.3 Hz, 2H, H_2), 1.72–1.52 (m, 4H, H_3 and H_5), 1.39 (m, 2H, H_4); ^{13}C NMR (75 MHz, $CDCl_3$) δ 179.04 (C_1), 158.39 (*C*ONH), 137.96 (q, $^2J_{C\text{-}F}$ = 35.5 Hz, C_3 Pz), 136.54 (C_5 Pz), 132.12 (C_3 Th), 130.71 (C_2 Th), 124.40 (C_4 Th), 121.49 (C_5 Th), 121.25 (q, $^1J_{C\text{-}F}$ = 269.1 Hz), 117.19 (C_4 Pz), 39.98 (N*C*H_3), 33.86 (C_2), 30.55 (C_6), 28.58 (C_5), 26.62 (C_4), 24.36 (C_3); ^{19}F NMR (282 MHz, DMSO-d_6) δ −60.13 (s); HRMS (ES) calculated for $C_{16}H_{19}F_3N_3O_3S$ $[M+H]^+$ 390.1094, found 390.1105. UV (PB) ε (280 nm) 1.50 mM^{-1} cm^{-1}.

Synthesis of 3-aminothiophene 5



Scheme S3

***N*-(Thiophen-3-yl)benzamide (16)**. Benzoyl chloride (1.2 mL, 10.02 mmol) was added drop wise to a suspension of methyl 3-aminotiophene-2-carboxylate (**15**, 1.5 g, 9.54 mmol) and triethylamine (1.6 mL, 11.26 mmol) in anhydrous CH_2Cl_2 (10 mL) at 5 °C under nitrogen. The resulting mixture was stirred at room temperature for 3 h (reaction monitored by TLC, CH_2Cl_2),

then diluted with CH_2Cl_2 and washed with 5% $NaHCO_3$ aqueous solution and brine, dried over anhydrous $MgSO_4$, filtered and concentrated under vacuum. The crude product was purified by flash column chromatography (silica gel, CH_2Cl_2), to give the corresponding benzoyl derivative of **15** as a pale yellow solid (2.409 g, 97%).

A suspension of the above compound (800.0 mg, 3.06 mmol) and pyridine hydrochloride (1.06 g, 9.19 mmol) in anhydrous pyridine (8.5 mL) was refluxed (130 °C) with stirring for 48 h under N_2 atmosphere (reaction monitored by TLC, CH_2Cl_2). After this time, the mixture was poured into ice, acidified with concentrated hydrochloric acid to pH 2, and then extracted with EtOAc. The combined organic layers were washed with brine, dried over anhydrous $MgSO_4$, filtered and concentrated under vacuum, to give compound **16** as a pale brown solid (602.1 mg, 97%). The crude compound thus obtained showed a purity >95 % by NMR and could be used without further purification for the next step.

Physical and spectroscopic data: Mp 145–146.8 °C ($CHCl_3$) [lit.,[3,7] mp 155.4–156.2 °C]; IR (KBr) ν_{max} (cm^{-1}) 3331.8w, 3139.6w, 3112.4w, 3051.6w, 2927.0w, 1651.5m, 1569.9m, 1284.3m, 771.2m; ^{1}H NMR (300 MHz, MeOH-d_4) δ 7.93–7.87 (m, 2H, H_2 and H_6 Ph), 7.69 (dd, J = 3.2, 1.3 Hz, 1H, H_2 Th), 7.58–7.43 (m, 3H, H_3, H_4 and H_5 Ph), 7.33 (dd, J = 5.2, 3.2 Hz, 1H, H_5 Th), 7.25 (dd, J = 5.2, 1.4 Hz, 1H, H_4 Th); ^{13}C NMR (75 MHz, MeOH-d_4) δ 167.93 (*C*ONH), 137.79 (C_3 Th), 135.81 (C_1 Ph), 132.85 (C_4 Ph), 129.64 (C_3 and C_5 Ph), 128.52 (C_2 and C_6 Ph), 125.17 (C_4 Th), 122.94 (C_5 Th), 111.75 (C_2 Th); HRMS (ES) calculated for $C_{11}H_{10}NOS$ $[M+H]^+$ 204.0478, found 204.0474.

***N*-(2-(4-Methylpent-2-en-2-yl)thiophen-3-yl)benzamide (17)**. 4-Methylpentan-2-one (1.02 mL, 8.12 mmol, 3 equiv.) was added to a suspension of **16** (550.0 mg, 2.71 mmol) and *p*-toluenesulfonic acid monohydrate (257.4 mg, 1.35 mmol) in toluene under nitrogen. The resulting mixture was heated to 130 °C for 5.5 h, and the formed water was removed using a Dean-Stark trap (reaction monitored by TLC, hexane/EtOAc 8:2). After cooling to room temperature the reaction mixture was diluted with benzene and then washed with 1 M NaOH aqueous solution. The organic layer was washed with brine and dried over anhydrous $MgSO_4$, then filtered and concentrated under vacuum. The crude product was purified by flash column chromatography (silica gel, hexane/EtOAc 95:5), to afford **17** as a 7:3 mixture of *E*,*Z* isomers as a yellowish solid (473.3 mg, 61%).

Physical and spectroscopic data: Mp 79.2–80.9 °C (hexane) [lit.,[3] colourless crystals]; IR (KBr) ν_{max} (cm^{-1}) 3269.6s, 2957.9s, 1649.1s, 1515.1s, 1489.7s, 1288.3s, 714.4m, 689.7m; ^{1}H NMR data of the major (*E*) isomer (300 MHz, $CDCl_3$) δ 8.27 (s, 1H, N*H*), 7.94 (d, J = 5.5 Hz,

[7] Daisuke Ura; Hiroyuki Katsuta; Toshio Kitashima; Kenichi Sato. Process for producing 2-alkyl-3-aminothiophene. WO2004009581 (A1), 2004-01-29.

1H, H_4 Th), 7.88–7.79 (m, 2H, H_2 and H_6 Ph), 7.58–7.44 (m, 3H, H_3 H_4 and H_5 Ph), 7.19 (dd, J = 5.5, 0.3 Hz, 1H, H_5 Th), 5.57 (dq, J = 9.3, 1.4 Hz, 1H, $H_{3'}$), 2.75 (d sept, J = 9.3, 6.7 Hz, 1H, $H_{4'}$), 2.05 (d, J = 1.4 Hz, 3H, $H_{1'}$), 1.07 (d, J = 6.7 Hz, 6H, $H_{5'}$ *and Me-4'*) ^{13}C NMR data of the major (*E*) isomer (75 MHz, $CDCl_3$) δ 164.11 (*C*ONH), 139.71 ($C_{3'}$), 134.71 (C_3 Th), 134.64, 131.93 (C_4 Ph), 131.33 (C_1 Ph), 128.91 (C_3 andC_5 Ph), 127.04 (C_2 and C_6 Ph), 126.44 ($C_{2'}$), 124.48 (C_2 Th), 123.10 (C_4 Th), 122.26 (C_5 Th), 28.10 ($C_{4'}$), 23.12 (Me-4' and $C_{5'}$), 19.04 ($C_{1'}$); HRMS (ES) calculated for $C_{17}H_{20}NOS$ $[M+H]^+$ 286.1260, found 286.1260.

***N*-(2-(4-Methylpentan-2-yl)thiophen-3-yl)benzamide (18)**. A suspension consisting of **17** (400.0 mg, 1.40 mmol) and 10% Pd/C (246.1 mg) in anhydrous MeOH (10.2 mL) was hydrogenated at room temperature for 3 days under a pressure of hydrogen of 5 bar, as described above for **7**. After this time, the black suspension was filtered through a pad of celite, eluting with methanol. The volatiles were removed under vacuum to give compound **18** as a white solid which required no further purification (382.2 mg, 95%).

Physical and spectroscopic data: Mp 104.1–105.0 °C ($CHCl_3$) [lit.,[3] colourless solid]; IR (KBr) ν_{max} (cm^{-1}) 3295.5s, 2958.1m, 2927.3w, 1647.8s, 1517.5m, 1485.9m, 1286.0m, 704.0m, 688.5w; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.85 (d, J = 7.1 Hz, 2H, H_2 and H_6 Ph), 7.65 (s, 1H, N*H*), 7.58–7.44 (m, 3H, H_3 H_4 and H_5 Ph), 7.41 (d, J = 5.3 Hz, 1H, H_4 Th), 7.13 (d, J = 5.4 Hz, 1H, H_5 Th), 3.13 (sext, J = 6.8 Hz, 1H, $H_{2'}$), 1.66–1.37 (m, 3H, $H_{3'}$ and $H_{4'}$), 1.30 (d, J = 6.8 Hz, 3H, $H_{1'}$), 0.89 (d, J = 6.3 Hz, 3H, $H_{5'}$), 0.88 (d, J = 6.2 Hz, 3H, *Me-4'*); ^{13}C NMR (75 MHz, $CDCl_3$) δ 165.51 (*C*ONH), 139.45 (C_3 Th), 134.73 (C_1 Ph), 131.90 (C_4 Ph), 130.35 (C_2 Th), 128.91 (C_3 and C_5 Ph), 127.15 (C_2 and C_6 Ph), 124.57 (C_4 Th), 121.15 (C_5 Th), 48.64 ($C_{3'}$), 30.57 ($C_{2'}$), 25.75 ($C_{4'}$), 23.09 ($C_{1'}$), 22.88 and 22.71 ($C_{5'}$ and Me-4'); HRMS (ES) calculated for $C_{17}H_{22}NOS$ $[M+H]^+$ 288.1417, found 288.1414.

2-(4-Methylpentan-2-yl)thiophen-3-amine (5). A mixture of KOH (1.43 g, 25.44 mmol), **18** (325.0 mg, 1.13 mmol) and diethylenglycol (34 mL) was stirred at 185 °C for 35 minutes under microwaves irradiation (300 W). After this time, the mixture was diluted in water and extracted with EtOAc. The combined organic layers were washed with brine and dried over anhydrous $MgSO_4$, then filtered and concentrated under vacuum. The crude product was purified by flash column chromatography (silica gel, hexane/CH_2Cl_2 3:7), to give **5** (119.8 mg, 58%).

Physical and Spectroscopic data: A yellowish oil [lit.,[3] yellow oil]. IR (KBr) ν_{max} (cm^{-1}) 3432.1m, 3348.0m, 2955.7s, 2926.0s, 2867.6m, 1655.5w, 1567.0s, 1459.0w, 1258.0w, 718.3m, 636.8m; ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.95 (dd, J = 5.3, 0.5 Hz, 1H, H_4 Th), 6.55 (d, J = 5.2 Hz, 1H, H_5 Th), 3.34 (br s, 2H, NH_2), 2.95 (sext, J = 6.8 Hz, 1H, $H_{2'}$), 1.69–1.35 (m, 3H, $H_{3'}$ and $H_{4'}$), 1.24

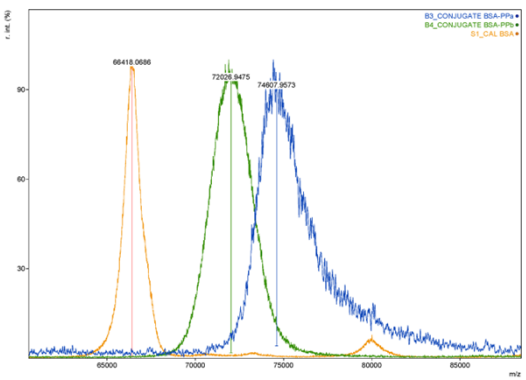
(d, J = 6.8 Hz, 3H, $H_{1'}$), 0.91 (d, J = 6.5 Hz, 3H, $H_{5'}$), 0.90 (d, J = 6.4 Hz, 3H, *Me-4'*); ^{13}C NMR (75 MHz, $CDCl_3$) δ 138.99 (C_3 Th), 125.48 (C_2 Th), 121.80 (C_4 Th), 120.70 (C_5 Th), 48.20 ($C_{3'}$), 29.83 ($C_{2'}$), 25.85 ($C_{4'}$), 22.85 (Me-4' and $C_{5'}$), 22.66 ($C_{1'}$); HRMS (GC–MS EI) calculated for $C_{10}H_{17}NS$ M^+ 183.1082, found 183.1075.

Hapten activation and conjugation

The hapten (1 equiv) and *N*,*N'*-disuccinimidyl carbonate (1.3 equiv) were dissolved in anhydrous acetonitrile (0.9 mL per 0.1 mmol of hapten) under nitrogen in an ice-water bath. Triethylamine (3.8 equiv) was then added, and the resulting mixture was stirred at room temperature until complete consumption of the starting material (as observed by thin layer chromatography using $CHCl_3$/MeOH 95:5 as eluent). The reaction mixture was diluted with ethyl ether, washed with a 5% aqueous solution of $NaHCO_3$ and brine, and dried over anhydrous $MgSO_4$. After evaporation of the solvent, the activated hapten was purified by column chromatography, using $CHCl_3$ as eluent.

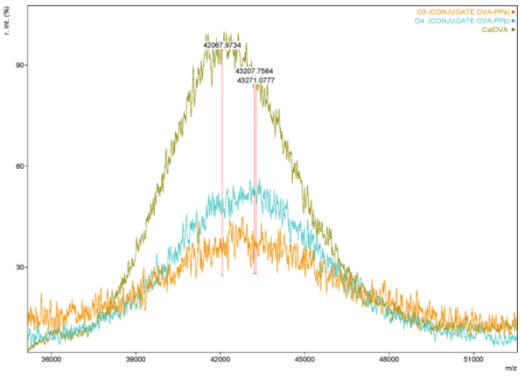
Conjugation was carried out in 50 mM carbonate buffer, pH 9.6, during 2 h under moderate stirring at room temperature. Immunizing conjugates were prepared by reaction of 10 µmol of purified activated hapten in DMF (200 µL) with 15 mg of BSA in carbonate buffer (1.8 mL). For coating conjugates, 5 µmol of activated hapten in DMF (100 µL) was conjugated to 15 mg of OVA in the described buffer (1.9 mL), whereas for enzyme assay conjugates, 0.5 µmol of active ester solution in DMF (100 µL) was reacted with 2.2 mg of HRP in carbonate buffer (0.9 mL). Conjugates were purified by gel filtration chromatography using 100 mM phosphate buffer, pH 7.4 as eluent. BSA and OVA conjugates were stored frozen at −20 °C, and HRP conjugates were kept at 4 °C.

Samples for molecular weight determination were at concentration of ca 0.3 µg/µL in water; 1 µL of every sample solution was spotted onto the MALDI plate, after the droplets were air-dried at room temperature, 0.75 µL of matrix [5 mg/mL sinapinic acid (Bruker) in 0.1% TFA-CH_3CN/H_2O (7:3, v/v)] was added and allowed to air-dry at room temperature. The resulting mixtures were analyzed in a 5800 MALDI TOF-TOF (ABSciex) in positive linear mode (1500 shots every position) in a mass range of 10000-120000 m/z. Previously, the Plate was calibrated with 1 µL 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.



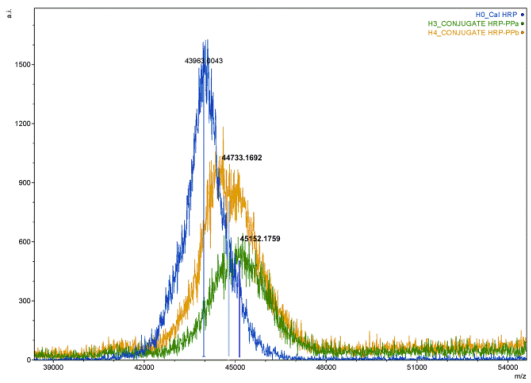
	m/z	*Δm/hapten*	*Δ(m/z)*	MR
BSA	66418			
BSA-PP*a*	74608	427,2	8190	19.2
BSA-PP*b*	72027	371,1	5609	15.1

Fig. S1. MALDI-TOF/MS spectra of BSA (orange) and BSA-hapten conjugates: BSA–PP*a* (blue) and BSA–PP*b* (green).



	m/z	*Δm/hapten*	*Δ(m/z)*	MR
OVA	42068			
OVA-PP*a*	43207	427-2	1139	2.7
OVA-PP*b*	43271	371.1	1203	3.2

Fig. S2. MALDI-TOF/MS spectra of OVA (olive green) and OVA-hapten conjugates: OVA–PP*a* (orange) and OVA–PP*b* (light blue).



	m/z	*Δm/hapten*	*Δ(m/z)*	MR
HRP	43963			
HRP-PP*a*	45182	427.2	1219	2.9
HRP-PP*b*	44773	371.1	810	2.2

Fig. S3. MALDI-TOF/MS spectra of HRP (blue) and HRP-hapten conjugates: HRP–PP*a* (green) and HRP–PP*b* (orange).

Antibody generation

Two antisera were generated with each immunogen 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–hapten 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 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 sulfate solution. Salting out was performed twice, and precipitates were stored at 4 °C.

Competitive ELISA

Antibody-coated direct cELISA

Coating for direct cELISAs was performed by overnight incubation at room temperature in sealed plates bearing 100 µL per well of antibody dilution in 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 penthiopyrad standard solution in PBS (10 mM phosphate, pH 7.4 containing 140 mM NaCl) and 50 µL per well of HRP conjugate 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.

Conjugate-coated indirect cELISA

Coating was carried out in sealed plates by incubation overnight at room temperature with 100 µL per well of OVA conjugate solution in coating buffer. After washing as described, the competitive step was done with 50 µL per well of penthiopyrad 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-labeled 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.

For all assays, absorbance was read immediately at 492 nm with a reference wavelength at 650 nm. Experimental values were fitted to a four-parameter logistic equation using the SigmaPlot software package from SPSS Inc. (Chicago, IL, USA).

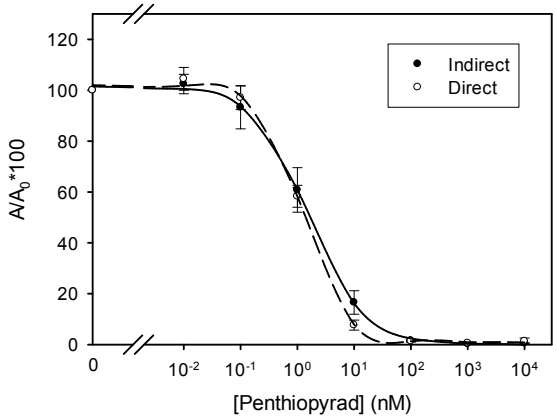
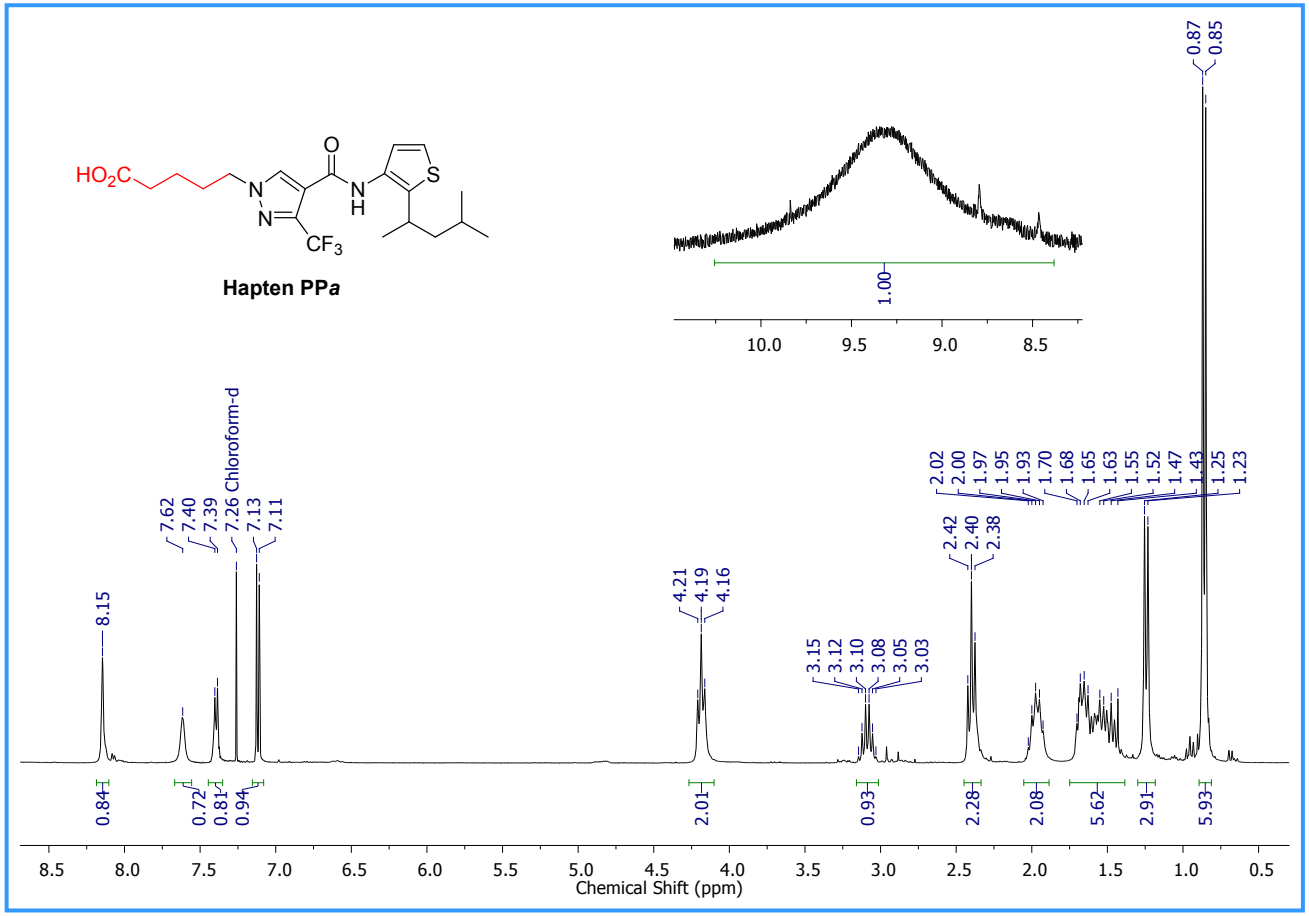


Fig. S4. Standard curves obtained with antibody PP*a*#2 in two cELISA formats.

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



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