SUPPLEMENTARY INFORMATION

Mepanipyrim haptens and antibodies with nanomolar affinity

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Experimental

Chemicals and instruments

tert-Butyl 6-iodohexanoate (**1**) and *tert*-butyl hex-5-ynoate (**6**) were prepared from commercial 6-bromohexanoic acid[1] and hex-5-ynoic acid,[2] respectively. Other reagents were obtained from commercial sources and used without purification. Reactions were monitored by thin layer chromatography (TLC) carried out on 0.25 mm Merck silica gel plates (60F-254) using UV light as the visualizing agent and ethanolic phosphomolybdic acid or aqueous ceric ammonium molybdate solutions and heat as developing agents. Merck silica gel (60, particle size 0.043–0.063 mm) was used for flash column chromatography. All melting points were determined using a Kofler hot-stage apparatus and are uncorrected. NMR spectra were acquired at room temperature (rt) on a Bruker AC-300 spectrometer (300.13 MHz for ^{1}H and 75.47 MHz for ^{13}C). The spectra were referenced to residual solvent protons in the ^{1}H NMR spectra ($CDCl_3$ = 7.26 and THF-d_6 = 3.58) and to solvent carbons in the ^{13}C NMR spectra ($CDCl_3$ = 77.0). Carbon substitution degrees were established by DEPT pulse sequences. Infrared (IR) spectra were obtained using a Nicolet Avatar 320 FT-IR spectrometer. High resolution mass spectra (HRMS)

were recorded by the electrospray (ES) ionization mode using a Micromass VG Autospec spectrometer.

Horseradish peroxidase (HRP), ovalbumin (OVA), and *o*−phenylenediamine were purchased from Sigma/Aldrich (Madrid, Spain). Sephadex G-25 HiTrap Desalting columns from GE Healthcare (Uppsala, Sweden) were used for conjugate purification in the ÄKTA workstation. Bovine serum albumin (BSA) fraction V was purchased from Roche Applied Science (Mannheim, Germany). Foetal bovine serum and Freund's adjuvants were also from Sigma/Aldrich. Costar flat-bottom high-binding polystyrene ELISA plates were from Corning (Corning, NY, USA). Standard stock solutions were prepared as concentrated solutions in anhydrous *N*,*N*-dimethylformamide (DMF) and kept at −20 ºC in amber glass vials.

Hapten synthesis

Synthesis of hapten MPn6.

Preparation of tert-butyl 6-((4-methyl-6-(prop-1-ynyl)pyrimidin-2-yl)(phenyl)amino) hexanoate (**2**). A solution of mepanipyrim (200 mg, 0.90 mmol) in anhydrous DMF (3 mL) was added dropwise to a suspension of prewashed (pentane) 60% NaH in mineral oil (39.5 mg, 0.99 mmol, 1.1 equiv.) in DMF (3 mL) at rt under nitrogen atmosphere. After foaming had subsided, iodide **1** (536 mg, 1.8 mmol, 2.0 equiv.) was added followed by stirring during 60 hours. The reaction was then diluted with water and extracted with EtOAc. The combined organic extracts were washed with an aqueous solution of LiCl and brine and dried over anhydrous Na_2SO_4. The solvent was removed and the residue purified by column chromatography, using hexane/EtOAc 9:1 as eluent, to afford *tert*-butyl ester **2** (173.2 mg, 49%) as an oil. ^{1}H NMR (300 MHz, $CDCl_3$) **δ** (ppm) 7.32 (2H, m, H-3 and H-5 Ph), 7.21 (2H, m, H-2 and H-6 Ph), 7.15 (1H, tt, *J* = 7.5 and 1.5 Hz, H-4 Ph), 6.49 (1H, s, H-5 Pym), 3.97 (2H, m, H-6), 2.26 (3H, s, Me-Pym), 2.19 (2H, t, *J* = 7 Hz, H-2), 1.99 (3H, s, Me-C≡C), 1.68-1.52 (4H, m, H-3 and H-5), 1.42 (9H, s, CMe_3), 1.33 (2H, m,

H-4); ^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm) 173.1 (C-1), 167.4 (C-2 Pym), 161.7 (C-4 Pym), 150.9 (C-6 Pym), 144.3 (C-1 Ph), 128.8 (C-3 and C-5 Ph), 127.5 (C-2 and C-6 Ph), 125.4 (C-4 Ph), 112.6 (C-5 Pym), 88.3 (C-2'), 79.9 (*C*Me$_3$), 78.2 (C-1'), 50.1 (C-6), 35.5 (C-2), 28.1 (*CMe*$_3$), 27.4 (C-5), 26.2 (C-4), 24.8 (C-3), 24.1 (*Me*-Pym), 4.5 (*Me*-C≡C); IR (NaCl) ν_{max}/cm^{-1} 2974, 2931, 2858, 2245, 1728, 1562, 1546, 1497, 1151; HRMS (TOF MS ES+) *m/z* calcd. for $C_{24}H_{32}N_3O_2$ $[M+H]^+$ 394.2495, found 394.2501.

Preparation of 6-((4-methyl-6-(prop-1-ynyl)pyrimidin-2-yl)(phenyl)amino)hexanoic acid (Hapten MPn6). Formic acid (2 mL) was added to *tert*-butyl ester **2** (80 mg, 0.20 mmol) at 0ºC and the resulting solution allowed to warm to rt and stirred for 3 hours. The formic acid was removed in the rotavapor and the residue was chromatographed on a silica gel column using $CHCl_3$ as the eluent to give hapten MPn6 (63.3 mg, 94%) as a white solid. Mp 109.6-110.5 ºC (crystallized by slow evaporation from a $CDCl_3$ solution); ^{1}H NMR (300 MHz, $CDCl_3$) **δ** (ppm) ^{1}H NMR (300 MHz, $CDCl_3$) **δ** (ppm) 7.36 (2H, m, H-3 and H-5 Ph), 7.26-7.16 (3H, H-2, H-4 and H-6 Ph), 6.48 (1H, s, H-5 Pym), 3.96 (2H, m, H-6), 2.33 (2H, t, *J* = 7.5 Hz, H-2), 2.23 (3H, s, Me-Pym), 2.02 (3H, s, Me-C≡C), 1.72-1.58 (4H, m, H-3 and H-5), 1.37(2H, m, H-4); ^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm) 178.9 (C-1), 167.5 (C-2 Pym), 161.7 (C-4 Pym), 150.9 8 (C-1 Ph), 144.3 (C-6 Pym), 128.9 (C-3 and C-5 Ph), 127.54 (C-2 and C-6 Ph), 125.5 (C-4 Ph), 112.7(C-5 Pym), 88.5 (C-2'), 79.1 (C-1'), 50.0 (C-6), 33.7 (C-2), 27.2 (C-5), 26.1 (C-4), 24.3 (C-3), 24.1 (*Me*-Pym), 4.5 (*Me*-C≡C); IR (KBr) ν_{max}/cm^{-1} 3500-2500, 3428, 3070, 2946, 2856, 2247, 1707, 1560, 1545, 1499, 1342, 1248, 949, 820, 694; HRMS (TOF MS ES+) *m/z* calcd. for $C_{20}H_{24}N_3O_2$ $[M+H]^+$ 338.1869, found 338.1869.

Synthesis of hapten MPm6.

Preparation of 2-chloro-4-methyl-6-(prop-1-ynyl)pyrimidine (**4**). A 25 mL Büchi tinyclave steel reactor containing dichloropyrimidine **3** (400 mg, 2.47 mmol), ICu (4.8 mg, 0.025 mmol, 1%) and $Pd(PPh_3)_2Cl_2$ (52 mg, 0.074 mmol, 3%) was purged with nitrogen and then Et_3N (11 mL) was added. The reactor was cooled to -78ºC and charged with propyne gas (Aldrich, about 1.5-2 mL of condensed propyne). The reactor was allowed to warm to rt and the dark grey reaction mixture was stirred during 72 hours. After this time the reactor was ventilated with nitrogen and the residue left treated with saturated aqueous NH_4Cl solution and extracted with $CHCl_3$. The combined organic layers were washed with brine, dried over anhydrous Na_2SO_4 and evaporated to give a solid that was purified by column chromatography, eluting with hexane/EtOAc mixtures from 95:5 to 8:2, to give C6-acetylenic pyrimidine **4** (284 mg, 69.3%) as a colorless solid.[3] Mp 124-125.5 ºC (crystallized from cold hexane/ether); ^{1}H NMR (300 MHz, $CDCl_3$) **δ** (ppm) 7.09 (1H, s, H-5 Pym), 2.50 (Me-Pym), 2.11 (3H, s, 3H, s, Me-C≡C); ^{13}C NMR (75 MHz, $CDCl_3$) **δ** (ppm) 170.5 (C-4 Pym), 160.9 (C-2 Pym), 153.0 (C-6 Pym), 121.0 (C-5 Pym), 93.9 (C-2'), 77.5 (C-1'), 23.8 (Me-Pym), 4.5 (*Me*-C≡C); IR (KBr) ν_{max}/cm^{-1} 3066, , 2920, 2252, 2227, 1577, 1506, 1355, 1248, 1194, 1050, 916, 892, 812, 764, 571; HRMS (TOF MS ES+) *m/z* calcd. for $C_8H_8{}^{35}ClN_2$ $[M+H]^+$ 167.0376, found 167.0377.

Preparation of tert-butyl 6-(3-nitrophenyl)hex-5-ynoate (**7**). A mixture of $(Ph_3P)_2PdCl_2$ (46.7 mg, 0.066 mmol), CuI (33.8 mg, 0.177 mmol), aryl iodide **5** (553 mg, 2.22 mmol) was purged by three cycles of vacuum and nitrogen. Anhydrous degassed DMF (1.8 mL) and *tert*-butyl hex-5-ynoate (**6**, 560 mg, 3.33 mmol) were added and the resulting heterogeneous mixture was sonicated in the ultrasonic bath for two minutes, followed by the addition of anhydrous Et_3N (1.8 mL). The bright-red solution obtained was stirred at room temperature for 1.5 hours, then poured into water and extracted with EtOAc. The combined organic layers were washed with an aqueous solution of LiCl and

brine, dried over anhydrous Na_2SO_4 and concentrated to dryness. Chromatography of the crude product with hexane/EtOAc 9:1 as eluent afforded aryl-alkyne **7** (568 mg, 88.5%). as a colorless oil. ^{1}H NMR (300 MHz, $CDCl_3$) **δ** (ppm) 8.20 (1H, dd, *J* = 2 and 2 Hz, H-2 Ph), 8.10 (1H, ddd, *J* = 8, 2, and 1 Hz, H-4), 7.66 (1H, ddd, *J* = 8, 1 and 1 Hz, H-6), 7.44 (1H, dd, *J* = 8 and 8 Hz, H-5 Ph), 2.48 (2H, t, *J* = 7.3 Hz, H-4), 2.39 (2H, t, *J* = 7.3 Hz, H-2), 1.89 (2H, q, *J* = 7.3, H-3), 1.44 (9H, s, CMe_3); ^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm) 172.4 (C-1), 148.1 (C-3 Ph), 137.4 (C-6), 129.3 (C-2 Ph), 126.5 (C-5 Ph), 125.7 (C-1 Ph), 122.5 (C-4 Ph), 92.4 (C-5), 80.5 (*C*Me_3), 79.2 (C-6), 34.4 (C-2), 28.2 (*CMe*$_3$), 23.9 (C-3), 18.9 (C-4); IR (NaCl) ν_{max}/cm^{-1} 3083, 2970, 2232, 1725, 1529, 1349, 1145, 734; 674 HRMS (TOF MS ES+) *m/z* calcd. for $C_{16}H_{20}NO_4$ $[M+H]^+$ 290.1392, found 290.1387.

Preparation of tert-butyl 6-(3-aminophenyl)hexanoate (**8**). A mixture of aryl-alkyne **7** (265.2 mg, 0.92 mmol) and 10% Pd/C (45 mg) in EtOAc (4.4 mL) was stirred under an atmosphere of hydrogen at 50 psi during 5 hours. The reaction mixture was filtered through a short silica-gel column using EtOAc as the eluent. The filtrate was concentrated to dryness to give aniline **8** (232 mg, 96%) as a colorless viscous oil. ^{1}H NMR (300 MHz, $CDCl_3$) **δ** (ppm) 7.06 (1H, m, H-5 Ph), 6.49-6.60 (3H, m, H-2, H-4 and H-6 Ph), 3.54 (2H, br s, NH_2), 2.52 (2H, t, *J* = 7.5 Hz, H-6), 2.21 (2H, t, *J* = 7.5 Hz, H-2), 1.61(4H, quint, *J* = 7.5 Hz, H-3 and H-5), 1.44 (9H, s, CMe_3), 1.35 (2H, m, H-4); ^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm) 173.4 (C-1), 146.4 (C-3 Ph), 144.0 (C-1 Ph), 129.2 (C-5 Ph), 118.9 (C-6 Ph), 115.3 (C-2 Ph), 112.7 (C-4 Ph), 80.1 (*C*Me_3), 35.8 (C-6), 35.6 (C-2), 31.1 (C-3), 28.8 (C-5), 28.2 (*CMe*$_3$), 25.1 (C-4); IR (NaCl) ν_{max}/cm^{-1} 3468, 3373, 3003m 2930, 1720, 1621, 1604, 1589, 1459, 1366, 1151, 778; HRMS (TOF MS ES+) *m/z* calcd. for $C_{16}H_{26}NO_2$ $[M+H]^+$ 264.1963, found 264.1958.

Preparation of tert-butyl 6-(3-((4-methyl-6-(prop-1-yn-1-yl)pyrimidin-2-yl)amino)phenyl) hexanoate (**9**). A solution of xantphos (31.0 mg, 0.054 mmol) and $Pd(OAc)_2$ (6.2 mg, 0.027 mmol) in degassed anhydrous dioxane (1.5 mL) was added via

cannula to a suspension of chloropyrimidine **4** (45 mg, 0.27 mmol), aniline **8** (106.5 mg, 0.40 mmol) and K_2CO_3 (534 mg, 3.9 mmol) in dioxane (2.2 mL) previously degassed by several freeze–pump–thaw cycles. Then, the mixture was stirred at 100 ºC under argon for 3 hours, cooled to rt and filtered through a short pad of celite using EtOAc as eluent. The reddish filtrate was evaporated under reduced pressure and the residue chromatographed on silica gel, using hexane/EtOAc mixtures from 9: 1 to 85:15, to afford compound **9** (83 mg, 78.1%) as a semisolid. ^{1}H NMR (300 MHz, $CDCl_3$) **δ** (ppm) 7.52 (1H, ddd, *J* = 8, 2 and 1 Hz, H-4 Ph), 7.35 (1H, dd, *J* = 2 and 2 Hz, H-2 Ph), 7.22 (1H, dd, *J* = 8 and 8 Hz, H-5 Ph), 7.07 (1H, br s, N-H), 6.83 (1H, ddd, *J* = 8, 2 and 1 Hz, H-6 Ph), 6.63 (1H, s, H-5 Pym), 2.60 (2H, t, *J* = 7.5 Hz, H-6), 2.38 (3H, s, Me-Pym), 2.21 (2H, t, *J* = 7.5 Hz, H-2), 2.08 (3H, s, Me-C≡C), 1.63 (4H, quint, *J* = 7.5 Hz, H-3 and H-5), 1.43 (9H, s, CMe_3), 1.35 (2H, m, H-4); ^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm) 173.2 (C-1), 168.2 (C-2 Pym), 159.8 (C-4 Pym), 151.3 (C-6 Pym), 143.3 (C-1 Ph), 139.4 (C-3 Ph), 128.7 (C-5 Ph), 122.5 (C-4 Ph), 119.1 (C-2 Ph), 116.5 (C-6 Ph), 114.0 (C-5 Pym), 89.7 (C-2'), 79.9 (*C*Me_3), 78.5 (C-1'), 35.7 (C-6), 35.5 (C-2), 30.9 (C-3), 28.7 (C-4), 28.1 (*CMe*$_3$), 24.9 (C-5), 24.1 (*Me*-Pym), 4.4 (*Me*-C≡C); IR (KBr) ν_{max}/cm^{-1} 3274, 2975, 2930, 2856, 2244, 1727, 1578, 1155; HRMS (TOF MS ES+) *m/z* calcd. for $C_{24}H_{32}N_3O_2$ $[M+H]^+$ 394.2495, found 394.2499.

Preparation of 6-(3-((4-methyl-6-(prop-1-yn-1-yl)pyrimidin-2-yl)amino)phenyl) hexanoic acid (Hapten MPm6). A solution of *tert*-butyl ester **9** (77 mg, 0.19 mmol) in formic acid (2.5 mL) was stirred at rt for 6 hours and then the excess of formic acid was removed using a rotary evaporator. The residue was dissolved in benzene and concentrated again to obtain a solid that was washed with ethyl ether and dried under vacuum to give nearly pure hapten MPm6 (60 mg, 91%) as a solid. Mp 178.5-179.4 ºC (crystallized by slow evaporation from a THF solution). ^{1}H NMR (300 MHz, THF-d_6) **δ** (ppm) 10.6 (1H, br s, CO_2H), 8.64 (1H, br s, N-H), 7.70 (1H, dd, *J* = 8, and 1.5 Hz, H-4 Ph), 7.50 (1H, br s, H-2 Ph), 7.11 (1H, dd, *J* = 8 and 8 Hz, H-5 Ph), 6.74 (1H, d, *J* = 8Hz, H-6 Ph), 6.59 (1H, s, H-5

Pym), 2.59 (2H, t, *J* = 7.5 Hz, H-6), 2.30 (3H, s, Me-Pym), 2.23 (2H, t, *J* = 7.5 Hz, H-2), 2.05 (3H, s, Me-C≡C), 1.73-1.60 (4H, m, H-3 and H-5), 1.44-1.36 (2H, m, H-4); ^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm) 168.6 (C-1), 161.5 (C-2 Pym), 152.4 (C-4 Pym), 143.7 (C-6 Pym), 141.9 (C-1 and C-3 Ph), 129.3 (C-5 Ph), 122.5 (C-4 Ph), 119.9 (C-2 Ph), 117.4 (C-6 Ph), 114.0 (C-5 Pym), 89.1 (C-2'), 79.9 (C-1'), 36.9 (C-6), 34.5 (C-2), 32.3 (C-5), 29.9 (C-4), 26.1 (C-3), 24.1 (*Me*-Pym), 4.0 (*Me*-C≡C); IR (KBr) ν_{max}/cm^{-1} 3425, 3289, 3136, 2926, 2855, 2246, 1698, 1548, 1492, 1372, 1184, 781; HRMS (TOF MS ES+) *m/z* calcd. for $C_{20}H_{24}N_3O_2$ $[M+H]^+$ 338.1869, found 338.1876.

Conjugate preparation

i) Activation of haptens MPn6 and MPm6. Activation of the free carboxylate group of haptens MPn6 and MPm6 with *N,N'*-disuccinimidyl carbonate (DSC) and purification of the corresponding succinimidyl active ester was done as follow: the hapten (31 mg, 0.10 mmol,) and DSC (33.3 mg, 0.13 mmol, 1.3 equiv.) were dissolved in dry acetonitrile (1.0 mL) under nitrogen atmosphere at 0 ºC and treated with anhydrous Et_3N (38.7 mg, 53.5 µL, 0.38 mmol, 3.8 equiv.). The reaction mixture was allowed to warm at rt and stirred until complete consumption of starting material (as observed by TLC). The mixture was diluted with $CHCl_3$, washed with saturated $NaHCO_3$ and brine, and dried over anhydrous Na_2SO_4. After evaporation of the solvent, the remaining residue was purified by column chromatography, using $CHCl_3$ as eluent, to afford the *N*-succinimidyl ester of the hapten (MPn6-NHS or MPm6-NHS) in high yield (85-90%) and purity, as evidenced by the corresponding ^{1}H NMR spectrum.

^{1}H NMR of succinimidyl ester MPn-NHS ($CDCl_3$, 300 MHz) **δ** (ppm): 7.36 (2H, m, H-3 and H-5 Ph), 7.27-7.27 (3H, m, H-2, H-4 and H-6 Ph), 6.48 (1H, s, H-5 Pym), 4.00 (2H, t, *J* = 7.5 Hz, H-6), 2.82 (4H, br s, $OCCH_2CH_2CO$), 2.60 (2H, t, *J* = 7.5 Hz, H-2), 2.24

(3H, s, Me-Pym), 2.02 (3H, s, Me-C≡C), 1.78 (2H, quint, *J* = 7.5 Hz, H-3), 1.66 (2H, quint, *J* = 7.5 Hz, H-5), 1.43 (2H, quint, *J* = 7.4 Hz, H-4).

^{1}H NMR of succinimidyl ester MPm-NHS ($CDCl_3$, 300 MHz) **δ** (ppm): 7.55 (1H, dd, *J* = 8 and 1.5 Hz, H-4 Ph), 7.35 (1H, dd, *J* = 1.5 and 1.5 Hz, H-2 Ph), 7.22 (1H, dd, *J* = 7.8 and 7.8 Hz, H-5 Ph), 7.12 (1H, br s, N-H), 6.83 (1H, ddd, *J* = 7.8, 1.5 and .1.5 Hz, H-6 Ph), 6.62 (1H, s, H-5 Pym), 2.83 (4H, br s, $OCCH_2CH_2CO$), 2.62 (2H, t, *J* = 7.5 Hz, H-6), 2.60 (2H, t, *J* = 7.5 Hz, H-2), 2.38 (3H, s, Me-Pym), 2.08 (3H, s, Me-C≡C), 1.78 (2H, quint, *J* = 7.5 Hz, H-5), 1.67 (2H, quint, *J* = 7.5 Hz, H-3), 1.48 (2H, m, H-4).

ii) Immunizing conjugates. BSA–hapten conjugates were prepared using 200 µL of 50 mM solutions of the *N*-succinimidyl ester of haptens MPn6 or MPm6 (10 µmol) in anhydrous DMF. Briefly, the dissolved active esters were added drop wise over 15 mg mL^{-1} BSA solutions (2 mL) in 50 mM carbonate–bicarbonate buffer, pH 9.6 (CB), and the mixtures were stirred at room temperature in amber glass vials during 4 h. The obtained conjugates were purified by Sephadex G-25 gel filtration using 100 mM phosphate, pH 7.4 (PB) as eluent, and each collected volume was brought to 30 mL with PB. Conjugate solutions were stored at −20 ºC.

iii) *Coating conjugates.* OVA–hapten conjugates were prepared in amber glass vials by adding drop wise 200 µL of 35 mM solutions of the *N*-succinimidyl ester of haptens MPn6 or MPm6 (7 µmol) in DMF to 2 mL of 15 mg mL^{-1} OVA solutions in CB. Reactions were stirred at room temperature for 2.5 h and conjugates were purified as before, brought to a concentration of 1 mg mL^{-1} with PB containing 0.01% (w/v) thimerosal, and stored at −20 ºC.

iv) Enzyme tracers. HRP–hapten conjugates were prepared by adding drop wise 100 µL of 5 mM solutions of the *N*-succinimidyl ester of haptens MPn6 or

MPm6 (0.5 µmol) in DMF to 1mL of 2.2 mg mL^{-1} HRP solutions in CB under moderate stirring. Coupling reactions were allowed to proceed during 4 h at room temperature in amber glass vials. Then, conjugates were purified by gel chromatography, and diluted 1/2 with PBS containing 1% (w/v) BSA and 0.01% (w/v) thimerosal. Several aliquots of each enzyme tracer conjugate were stored at −20 ºC in amber vials, except the working solutions that were kept unfrozen at 4 ºC.

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