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Design and development of heterologous competitive immunoassays for the determination of boscalid residues

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SYNTHESIS OF BOSCALID HAPTENS BL*b* and BL*c*

General

All reactions involving air-sensitive compounds were conducted in oven-dried glassware under a nitrogen atmosphere. All solvents were purified by distillation and, if required, they were dried according to standard methods. *tert*-Butyl hex-5-ynoate (**3**) was prepared as described by Bartoli,[1] and *tert*-butyl 5-bromopentanoate (**13**) was synthesized as described by Mercader.[2] Compounds 1,3-diodobenzene (**1**), 4-chlorophenylboronic acid (**5**), 2-chloronicotinoyl chloride (**9**), 4-mercaptophenylboronic acid (**11**), 1-iodo-2-nitrobenzene (**15**), and all the other reagents used for the synthesis of the haptens were acquired from commercial sources and used without purification. The reactions were monitored with the aid of thin-layer chromatography (TLC) using 0.25 mm pre-coated silica gel plates. Visualization was carried out with UV light and aqueous ceric ammonium molybdate solution. Chromatography refers to flash column chromatography and it was carried out with the indicated solvents on silica gel 60 (particle size 0.040–0.063 mm). All melting points were determined using a Kofler hot-stage apparatus or a Büchi melting point apparatus and are uncorrected. All NMR spectra were recorded in $CDCl_3$ at room temperature on a Bruker AC-300 spectrometer (300.13 MHz for ^1H and 75.47 MHz for ^{13}C). The spectra were referenced to residual solvent protons in the ^1H NMR spectra (7.26 ppm) and to solvent carbons in the ^{13}C NMR spectra (77.0 ppm). Infrared (IR) spectra were measured as thin films between NaCl plates for liquid compounds and as KBr pellets for solids using a

Nicolet Avatar 320 spectrometer. High-resolution mass spectra (HRMS) were run either by the electron impact (EI, 70 eV) or fast atom bombardment (FAB), both obtained with a Micromass VG Autospec spectrometer, or the electrospray (ESI) mode, which was obtained with a Q-TOF premier mass spectrometer with an electrospray source (Waters, Manchester, UK).

Synthesis of Hapten BL*b*

Preparation of *2,4-diiodo-1-nitrobenzene* (**2**). 1,3-Diodobenzene (**1**) (2.15 g, 6.55 mmol) was added in small portions to fuming HNO_3 (10 mL) cooled at 0 ºC. Once the addition had finished, the reaction mixture was stirred for 15 min at the same temperature, carefully poured into water and extracted with CH_2Cl_2. The organic extracts were washed successively with saturated $NaHCO_3$ solution, aqueous 5% $NaHSO_3$ solution, and brine. Drying over anhydrous $MgSO_4$ and removal of solvent gave a yellow solid that was purified by flash chromatography, using hexane-EtOAc 95:5 as eluent, to give the nitro derivative **2** (2.36 g, 94%) as a crystalline yellow solid. Mp 95-97 ºC (from hexane) [Lit[3] 93-95 ºC]; IR ν_{max}/cm^{-1} (KBr), 3066, 1551, 1513, 1444, 1342; ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.43 (1H, d, *J* = 1.8 Hz, H-3), 7.82 (1H, dd, *J* = 8.5, 1.8 Hz, H-5), 7.60 (1H, d, *J* = 8.5 Hz, H-6); ^{13}C NMR (75 MHz, $CDCl_3$) δ 149.70 (C-3), 139.98 (C-1), 138.19 (C-5), 126.39 (C-6), 100.18 (C-4), 87.59 (C-2); MS (EI) *m/z* 375 (M^+, 97), 345 (17), 329 (19), 218 (21), 202 (30), 76 (7), 75 (100), 74 (46), 63 (15); HRMS (EI), calcd for $C_6H_3I_2NO_2$ 374.82533, found 374.82459.

Preparation of tert-butyl 6-(3-iodo-4-nitrophenyl)hex-5-ynoate (**4**). A solution of *tert*-butyl hex-5-ynoate (**3**) (295 mg, 1.75 mmol) in anhydrous degassed DMF (4 mL) was added to a mixture of diiodide **2** (438 mg, 1.17 mol), CuI (2.2 mg, 0.01 mmol) and $(Ph_3P)_2PdCl_2$ (24.6 mg, 0.03 mmol) followed by anhydrous Et_3N (3.1 mL) under nitrogen atmosphere. The reaction mixture, initially yellow that turned deep-orange after a few minutes, was stirred at rt for 22 h. The reaction mixture was transferred to a round bottom flask for evaporation of the Et_3N in a rotary evaporator and the residue was dissolved in EtOAc, washed with water and brine, dried over anhydrous Na_2SO_4, and concentrated. Chromatography of the crude product with hexane-EtOAc 8:2 as eluent afforded aryl-alkyne **4** (282 mg, 59%) as a yellow oil. IR ν_{max}/cm^{-1} (KBr)

2975, 2225, 1727, 1581, 1522, 1340, 1148; ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.05 (1H, d, *J* = 1.7 Hz, H-2 Ph), 7.81 (1H, d, *J* = 8.4 Hz, H-5 Ph), 7.44 (1H, dd, *J* = 8.4, 1.7 Hz, H-6 Ph), 2.49 (2H, t, *J* = 7.1 Hz, H-4), 2.39 (2H, t, *J* = 7.4 Hz, H-2), 1.89 (2H, quint, *J* = 7.2 Hz, H-3), 1.45 (9H, s, Me); ^{13}C NMR (75 MHz, $CDCl_3$) δ 172.19 (C-1), 151.26 (C-4 Ph), 144.57 (C-2 Ph), 131.76 (C-6 Ph), 129.90 (C-1 Ph), 125.22 (C-5 Ph), 95.56 (C-5), 86.12 (C-3 Ph), 80.51 (C-6), 78.31 (*C*Me$_3$), 34.32 (C-2), 28.10 (Me), 23.66 (C-3), 18.91 (C-4); MS (EI) *m/z* 415 (M^+, 0.2), 359 (32), 342 (29), 316 (15), 144 (13), 141 (15), 115 (14), 113 (19), 63 (11), 57 (100); HRMS (EI), calcd for $C_{16}H_{18}INO_4$ 415.02806, found 415.02812.

Preparation of tert-butyl 6-(4'-chloro-6-nitrobiphenyl-3-yl)hex-5-ynoate (**6**). A mixture of aryliodide **4** (238 mg, 0.56 mmol), 4-chlorophenylboronic acid (**5**) (107.4 mg, 0.68 mmol), K_3PO_4 (364 mg, 1.72 mmol), and $Pd(PPh_3)_4$ (26.4 mg, 0.024 mmol) in a previously degasified mixture of dioxane (2.9 mL) and water (0.6 mL) was stirred under nitrogen at 85 ºC for 20 h. After this time, the reaction mixture was cooled down, diluted with water, and extracted with EtOAc. The extracts were washed with brine, dried over anhydrous $MgSO_4$, and concentrated. Flash chromatography on silica gel eluting with hexane–EtOAc 98:2 afforded the biaryl compound **6** (190 mg, 83%) as a yellow oil. IR ν_{max}/cm^{-1} (NaCl) 2978, 2923, 2230, 1727, 1578, 1524, 1366, 1347, 1090, 837; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.84 (1H, d, *J* = 8.4 Hz, H-5 PhPh), 7.47 (1H, dd, *J* = 8.4, 1.8 Hz, H-4 PhPh), 7.40 (1H, d, *J* = 1.8 Hz, H-2 PhPh), 7.40 (2H, m, H-2'/H-6' PhPh), 7.23 (2H, m, H-3'/H-5' PhPh), 2.50 (2H, t, *J* = 7.0 Hz, H-4), 2.39 (2H, t, *J* = 7.4 Hz, H-2), 1.90 (2H, quint, *J* = 7.1 Hz, H-3), 1.45 (9H, s, CMe$_3$); ^{13}C NMR (75 MHz, $CDCl_3$) δ 172.24 (C-1), 147.24 (C-6 PhPh), 135.54 and 135.43 (C-1 and C-1' PhPh), 134.82 (C-2 PhPh), 134.57 (C-3 PhPh), 131.29 (C-4 PhPh), 129.19 (C-3'/ C-5' PhPh), 128.89 (C-2'/C-6' PhPh), 124.51 (C-5 PhPh), 94.51 (C-5), 80.49 (C-6), 79.43 (*C*Me$_3$), 34.36 (C-2), 28.10 (C*Me*$_3$), 23.77 (C-3), 18.91 (C-4); MS (EI) m/z 345 (17), 344 (11), 343 (48), 328 (8), 327 (5), 326 (21), 315 (6), 301 (8), 298 (7), 57 (100); HRMS (FAB), calcd for $C_{22}H_{23}{}^{35}ClNO_4$ (M^++1) 400.13156, found 400.13013.

Preparation of tert-butyl 6-(4'-chloro-6-nitrobiphenyl-3-yl)hexanoate (**7**). A solution of alkyne **6** (166.8 mg, 0.42 mmol) and Wilkinson catalyst (12 mg, 0.013 mmol, 3%) in THF (2.7 mL) was evacuated and purged under an

atmosphere of hydrogen gas. Then the hydrogen pressure was regulated to 4 atm and the reaction mixture was stirred at rt for 18 hours, the solvent was removed in vacuum, and the residue was purified by chromatography using hexane-EtOAc 95:5 as eluent to furnish the compound **7** (159.4 mg, 95%). IR ν_{max}/cm^{-1} (NaCl) 2976, 2934, 2861, 1728, 1585, 1522,1351, 1156; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.84 (1H, d, *J* = 8.3 Hz, H-5 PhPh), 7.40 (2H, H-2'/H-6' PhPh), 7.30 (1H, dd, *J* = 8.3, 1.9 Hz, H-4 PhPh), 7.25 (2H, m, H-3/H-5' PhPh), 7.19 (1H, d, *J* = 1.8 Hz, H-2 PhPh), 2.72 (2H, t, *J* = 7.6 Hz, H-6), 2.22 (2H, t, *J* = 7.3 Hz, H-2), 1.66 (4H, m, H-3 and H-5), 1.42 (9H, s, CMe_3), 1.37 (2H, m, H-4); ^{13}C NMR (75 MHz, $CDCl_3$) δ 172.92 (C-1), 148.19 (C-6 PhPh), 146.76 (C-3 PhPh), 136.31 (C-1' PhPh), 135.39 (C-4' PhPh), 134.16 (C-1 PhPh), 131.76 (C-2 PhPh), 129.22 (C-3'/C-5' PhPh), 128.72 (C-2'/C-6' PhPh), 128.32 (C-4 PhPh), 124.53 (C-5 PhPh), 80.02 (*C*Me_3), 35.57 (C-6), 35.26 (C-2), 30.59 (C-5), 28.49 (C-4), 28.03 (C*Me*$_3$), 24.70 (C-3); ESI-HRMS, calcd for $C_{22}H_{26}{}^{35}ClNNaO_4$ $[M+Na]^+$ 426.1448, found 426.1454.

Preparation of tert-butyl 6-(6-amino-4'-chlorobiphenyl-3-yl)hexanoate (**8**). A solution of nitro-biphenyl **7** (164 mg, 0.40 mmol) in 95% EtOH (2.6 mL) was added to a mixture of NH_4Cl (17 mg, 0.32 mmol) and iron powder (108.6 mg, 1.94 mmol) in water (0.8 mL). The mixture was stirred under reflux for 1 h, cooled to rt, and filtered. The filtrate was diluted with water and extracted with EtOAc. The extracts were washed with brine, dried over anhydrous Na_2SO_4, and concentrated to give aminobiphenyl **8** (149 mg, 98%) as a semisolid, which was rather pure as shown by NMR spectroscopy and was used without further purification in the next step. IR ν_{max}/cm^{-1} (NaCl) 3500-3300, 2930, 1727, 1618, 1485, 1366, 1155; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.40 (4H, br s, H-2'/H-6' and H-3'/H-5' PhPh,), 6.97 (1H, dd, *J* = 8.0, 2.0 Hz, H-4 PhPh), 6.91 (1H, d, *J* = 2.0 Hz, H-2 PhPh), 6.71 (1H, d, *J* = 8.0 Hz, H-5 PhPh), 2.54 (2H, t, *J* = 7.7 Hz, H-2), 2.22 (2H, t, *J* = 7.5 Hz, H-6), 1.62 (4H, m, H-3 and H-5), 1.44 (9H, s, CMe_3), 1.38 (2H, m, H-4); ^{13}C NMR (75 MHz, $CDCl_3$) δ 173.15 (C-1), 140.91 (C-6 PhPh), 138.04 (C-1' PhPh), 133.05 and 132.91 (C-3 and C-4' PhPh), 130.40 (C-2'/C-6' PhPh), 130.09 (C-2 PhPh), 128.83 (C-3'/C-5' PhPh), 128.64 (C-4 PhPh), 126.34 (C-1 PhPh), 115.93 (C-5 PhPh), 79.86 (*C*Me_3), 35.46 (C-6), 34.76 (C-2), 31.33 (C-5), 28.64 (C-4), 28.05 (C*Me*$_3$), 25.39 (C-3); MS (EI) *m/z* 376 (M^++1,

^{37}Cl, 2.3), 375 (M^+, ^{37}Cl, 10.9), 374 (M^++1, ^{35}Cl, 7.4), 373 (M^+, ^{35}Cl, 30.0), 318 (16), 317 (18), 316 (38), 300 (16), 218 (33), 217 (16), 216 (100), 57 (30); HRMS (EI), calcd for $C_{22}H_{28}{}^{35}ClNO_2$ 373.18086, found 373.17950.

Preparation of tert-butyl 6-(4'-chloro-6-(2-chloronicotinamido)biphenyl-3-yl)hexanoate (**10**). A solution of aminobiphenyl **8** (67 mg, 0.18 mmol) and nicotinoyl chloride (**9**) (34.7 mg, 0.20 mmol) in anhydrous THF (360 µL) was stirred at rt for 24 h. Thereafter the solvent was removed under vacuum and the residue was purified by chromatography, eluting with $CHCl_3$-hexane 1:1, to give amide **10** (87 mg, 95%) as a viscous oil. IR ν_{max}/cm^{-1} (NaCl) 3400-3150, 2977, 2932, 1727, 1674, 1520, 1398, 1154; ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.44 (1H, dd, *J* = 4.7, 1.9 Hz, H-6 Py), 8.26 (1H, d, *J* = 8.4 Hz, H-5 PhPh), 8.12 (1H, dd, *J* = 7.8, 1.9 Hz, H-4 Py), 8.06 (1H, s, NH), 7.44-7.30 (5H, m, H-2'/H-6', H-3'/H-5' and H-5 Py), 7.26 (1H, dd, *J* = 8.3, 1.8 Hz, H-4 PhPh), 7.07 (1H, d, *J* = 2.1 Hz, H-2 PhPh), 2.64 (2H, t, *J* = 7.7 Hz, H-6), 2.21 (2H, t, *J* = 7.3 Hz, H-2), 1.63 (4H, m, H-3 and H-5), 1.43 (9H, s, CMe_3), 1.32 (2H, m, H-4); ^{13}C NMR (75 MHz, $CDCl_3$) δ 173.08 (C-1), 162.46 (CON), 151.12 (C-6 Py), 146.66 (C-2 Py), 139.94 (C-1' PhPh), 139.90 (C-4 Py), 136.49 (C-3 Py), 134.17, 132.45, 131.83 and 131.15 (C-1, C-3, C-4' and C-6 PhPh), 130.70 (C-3'/C-5' PhPh), 130.05 (C-2 PhPh), 129.11 (C-2'/C-6'PhPh), 128.67 (C-4 PhPh), 122.79 (C-5 Py), 122.38 (C-5 PhPh), 79.94 (*C*Me_3), 35.39 (C-6), 35.11 (C-2), 30.99 (C-5), 28.59 (C-4), 28.04 (C*Me*$_3$), 24.83 (C-3); HRMS (EI), calcd for $C_{28}H_{30}{}^{35}Cl_2N_2O_3$ 512.16335, found 512.16444.

Preparation of 6-(4'-chloro-6-(2-chloronicotinamido)biphenyl-3-yl)hexanoic acid, Hapten BL*b*). A solution of *tert*-butyl ester **10** (68 mg, 0.13 mmol) in HCOOH (2.7 mL) was stirred at rt for 2 h. The reaction mixture was diluted with benzene and washed with water and brine, dried over anhydrous Na_2SO_4, and concentrated to dryness, to give hapten BLb6 (58 mg, 99%) as a white solid. Mp 137-139 ºC (from hexane-EtOAc); IR ν_{max}/cm^{-1} (KBr) 3500-2700, 3411, 3240, 2927, 2858, 1724, 1650, 1522, 1402; ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.33 (1H, dd, *J* = 4.7, 1.9 Hz, H-6 Py), 8.13 (1H, d, *J* = 8.3 Hz, H-5 PhPh), 8.01 (1H, dd, *J* = 7.6, 2.0 Hz, H-4 Py), 8.00 (1H, s, NH), 7.35-7.12 (5H, m, H-2'/H-6', H-3'/H-5, H-5 Py), 7.15 (1H, dd, *J* = 8.3, 1.8 Hz, H-4 PhPh), 6.98 (1H, d, *J* = 1.9 Hz, H-2 PhPh), 2.55 (2H, t, *J* = 7.5 Hz, H-6), 2.25 (2H, t, *J* = 7.4 Hz, H-2), 1.57

(4H, m, H-3 and H-5), 1.32 (2H, m, H-4); ^{13}C NMR (75 MHz, $CDCl_3$) δ 179.32 (C-1), 162.51 (CON), 151.17 (C-6 Py), 146.65 (C-2 Py), 140.03 (C-4 Py), 139.86 (C-1' PhPh), 136.44 (C-3 Py), 134.22, 132.53, 131.85 and 131.12 (C-1, C-3, C-4' and C-6 PhPh), 130.71 (C-3'/C-6'PhPh), 130.08 (C-2 PhPh), 129.15 (C-2'/C-6' PhPh), 128.70 (C-4 PhPh), 122.86 (C-5 Py), 122.45 (C-5 PhPh), 35.07 (C-6), 33.92 (C-2), 30.93 (C-5), 28.56 (C-4), 24.47 (C-3); HRMS (EI), calcd for $C_{24}H_{22}{}^{35}Cl_2N_2O_3$ 456.10075, found 456.10005. UV (100 mM sodium phosphate buffer, pH 7.4) Є (280 nm) 4.15 mM^{-1} cm^{-1}.

Synthesis of Hapten BL*c*

Preparation of 4-(5-tert-butoxy-5-oxopentylthio)phenylboronic acid (**14**). A mixture of boronic acid **11** (152 mg, 0.99 mmol), ethylene glycol (62 mg, 1 mmol), and anhydrous $MgSO_4$ (240 mg) in dry Et_2O (4 mL) was stirred at rt for 1 h. The reaction mixture was filtered through a plug of cotton and evaporated to obtain the silica gel-labile boronate ester **12** (168 mg, 93%) as a white solid, which was pure as shown by ^{1}H NMR spectroscopy. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.66 (2H, d, *J* = 8.4 Hz, H-3/H-5), 7.26 (2H, d, *J* = 8.4 Hz,, H-2/H-6), 4.36 (4H, s, OCH_2CH_2O).

A mixture of the above-obtained crude boronate ester (168 mg, 0.93 mmol), K_2CO_3 (193.5 mg, 1.4 mmol), NaI (40 mg, 0.27 mmol), and *tert*-butyl 5-bromopentanoate (**13**) (332 mg, 1.4 mmol) in dry CH_3CN (3.4 mL) was stirred at rt for 5 h. The reaction mixture was diluted with Et_2O, washed with brine, and dried over anhydrous $MgSO_4$. Evaporation of the solvent under vacuum left a residue that was adsorbed on silica gel and deposited on the top of a silica gel column. Elution with $CHCl_3$ afforded alkylthioaryl boronic acid **14** (267 mg, 85%) as an amorphous solid. IR ν_{max}/cm^{-1} (KBr) 3433, 2976, 2932, 1728, 1594, 1397, 1367, 1156, 1103, 1015, 741; ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.09 (2H, d, *J* = 8.1 Hz, H-2/H-6 Ph), 7.37 (2H, d , *J* = 8.1 Hz, H-3/ H-5 Ph), 3.02 (2H, t, *J* = 6.7 Hz, H-1), 2.26 (2H, t, *J* = 6.8 Hz, H-4), 1.76 (4H, m, H-2 and H-3), 1.43 (9H, s, CMe_3); ^{13}C NMR (75 MHz, $CDCl_3$) δ 172.66 (C-5), 143.11 (C-4 Ph), 135.92 (C-2/C-6 Ph), 126.47 (C-3/C-5 Ph), 80.27 (*C*Me_3), 35.00 (C-1), 31.78 (C-4), 28.33 (C-2), 28.08 (*C*Me_3), 24.32 (C-3); ESI--HRMS, calcd for $C_{15}H_{23}BNaO_4S$ 333.13078, found 333.13046.

Preparation of tert-butyl 5-(2'-nitrobiphenyl-4-ylthio)pentanoate (**16**). $Pd(PPh_3)_4$ (18 mg, 0.015 mmol) was added under nitrogen to a mixture of boronic acid **14** (123 mg, 0.40 mmol), 1-iodo-2-nitrobenzene (**15**) (118.5 mg, 0.47 mmol), and K_3PO_4 (260 mg, 1.22 mmol) in a mixture of dioxane (2 mL) and water (0.4 mL). The resulting mixture was stirred at reflux for 18 h, cooled to rt and diluted with Et_2O. The diluted mixture was then washed with water and brine, dried over anhydrous $MgSO_4$, and concentrated under reduced pressure to yield yellowish oil that was further purified by chromatography, using hexane-Et_2O mixtures (from 9:1 to 8:2) as eluent, to afford the biaryl derivative **16** (132 mg, 86%) as an oil. IR ν_{max}/cm^{-1} (NaCl) 2976, 2932, 2867, 1726, 1608, 1529, 1365, 1156, 853, 751; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.84 (1H, dd, *J* = 8.0, 1.5 Hz, H-3' PhPh), 7.60 (1H, ddd, *J* = 9.0, 8.0, 1.5 Hz, H-5' PhPh), 7.47 (1H, ddd, *J* = 9.0, 8.0, 1.5 Hz, H-4' PhPh), 7.42 (1H, dd, *J* = 8.0, 1.5 Hz, H-6' PhPh), 7.34 (2H, m, H-2 and H-5 PhPh), 7.22 (2H, m, H-3 and H-5 PhPh), 2.97 (2H, t, *J* = 7.0 Hz, H-5), 2.26 (2H, t, *J* = 7.0 Hz, H-2), 1.73 (4H, m, H-3 and H-4), 1.44 (9H, s, CMe_3); ^{13}C NMR (75 MHz, $CDCl_3$) δ 172.66 (C-1), 149.17 (C-1' PhPh), 137.51 (C-2' PhPh), 135.66 and 134.47 (C-1 and C-4 PhPh), 132.28 and 131.81 (C-5' and C-6'), 128.37 and 128.31 (C-2/C-6 and C-3/C-5 PhPh), 128.12 (C-4' PhPh), 124.11 C-3' PhPh), 80.21 (*C*Me$_3$), 34.99 (C-5), 32.73 (C-2), 28.41 (C-4), 28.07 (C*Me*$_3$), 24.25 (C-3); MS (EI) *m/z* 387 (M^+, 31), 332 (11), 331 (53), 315 (15), 314 (74), 312 (37), 286 (17), 270 (94), 231 (37), 57 (100); HRMS (EI), calcd for $C_{21}H_{25}NO_4S$ 387.15043, found 387.15015.

Preparation of tert-butyl 5-(2'-aminobiphenyl-4-ylthio)pentanoate (**17**). A solution of NH_4Cl (8.9, 0.17 mmol) in water (0.4 mL) was added dropwise to a solution of nitro-biphenyl **16** (130 mg, 0.33 mmol) in EtOH (1.3 mL). Iron powder (60 mg, 1.0 mmol) was added in small portions to the resulting turbid solution and the reaction mixture was heated at reflux with stirring for 1 h, cooled to rt and filtered. The filtrate was diluted with benzene and concentrated to dryness. The residue obtained was purified by chromatography, using hexane-Et_2O 6:4 as eluent, to afford the aminobiphenyl **17** (105 mg, 90%) as a colorless oil. IR ν_{max}/cm^{-1} (NaCl) 3466, 3373, 2976, 2931, 1726, 1614, 1482, 1368, 1156, 750; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.38 (4H, br s, H-2/H-6 and H-3/H-5 PhPh), 7.15 (1H, ddd, *J* = 9.0, 7.9, 1.5 Hz, H-4' PhPh), 7.10 (1H, ddd, *J* = 7.5, 1.5 Hz, H-6'

PhPh), 6.82 (1H, ddd, *J* = 9.0, 7.5, 1.0 Hz, H-5' PhPh), 6.76 (1H, ddd, *J* = 7.9, 1.0 Hz, H-3' PhPh), 3.72 (2H, br s, NH_2), 2.97 (2H, t, *J* = 6.9 Hz, H-5), 2.26 (2H, t, *J* = 6.9 Hz, H-2), 1.74 (4H, m, H-3 and H-4), 1.44 (9H, s, CMe_3); ^{13}C NMR (75 MHz, $CDCl_3$) δ 172.65 (C-1), 143.43 (C-2' PhPh), 136.93 and 135.55 (C-1 and C-4 PhPh), 130.29 (C-4'), 129.50 and 129.09 (C-2/C-6 and C-3/C-5 PhPh), 128.49 (C-6' PhPh), 126.87 (C-1' PhPh), 118.64 (C-5'), 115.60 (C-3' PhPh), 80.17 (CMe_3), 34.99 (C-5), 33.10 (C-2), 28.53 (C-4), 28.05 (CMe_3), 24.24 (C-3); MS (EI) *m/z* 357

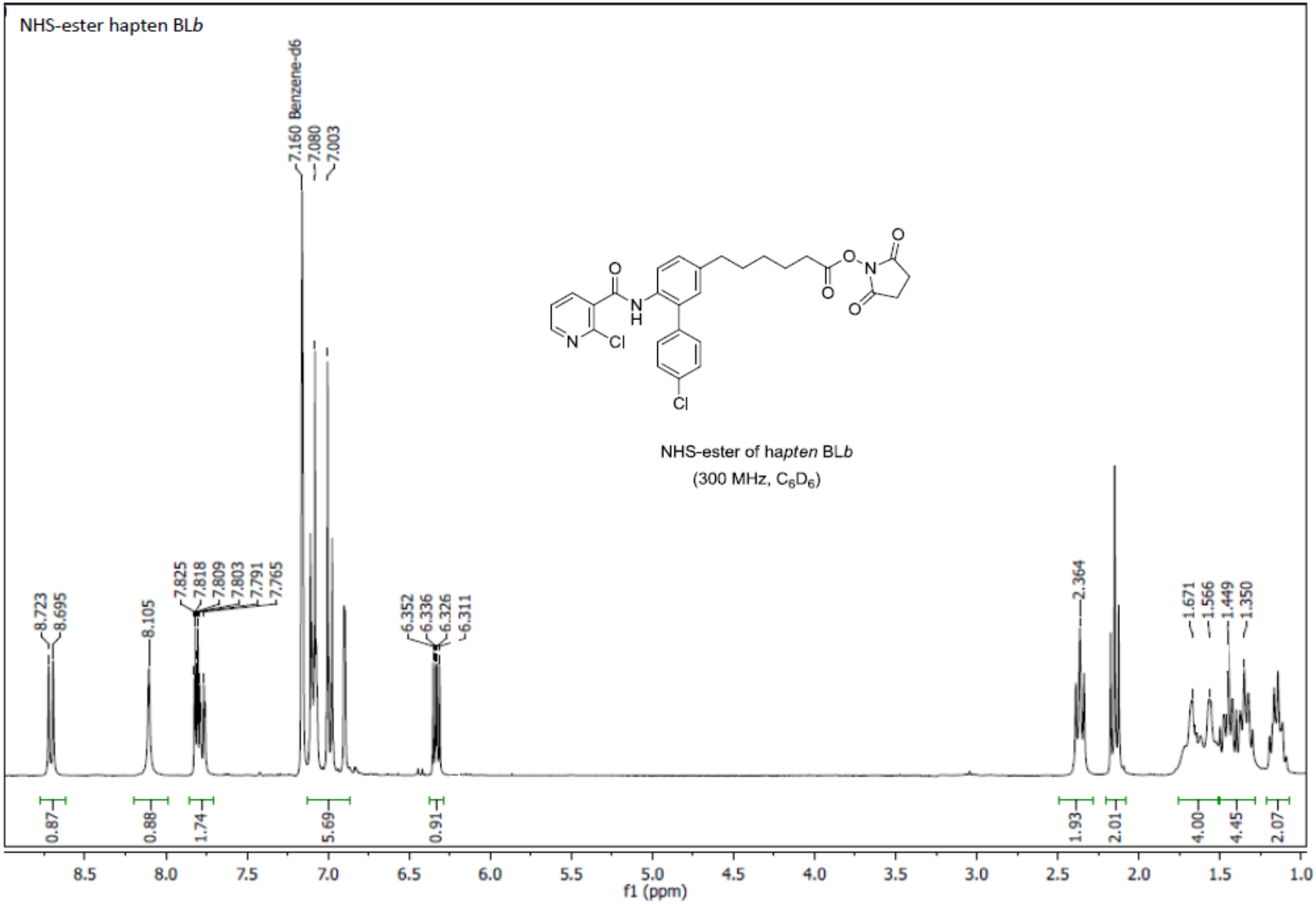
(M^+, 88), 326 (7), 324 (2), 303 (16), 301 (78), 284 (33), 240 (15), 201 (54), 57 (100); HRMS (EI), calcd for $C_{21}H_{27}NO_2S$ 357.17625, found 357.17558.

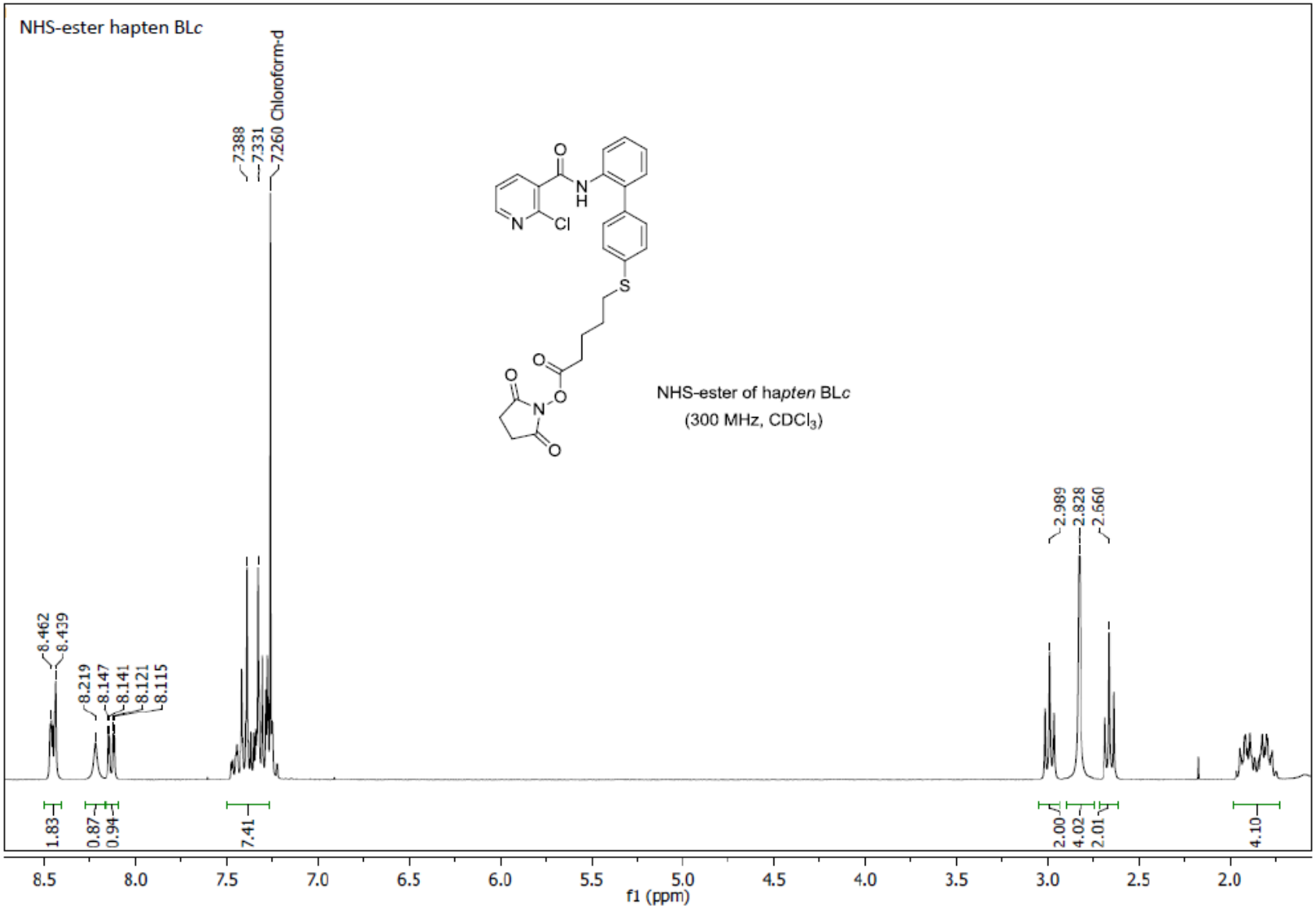
Preparation of tert-butyl 5-(2'-(2-chloronicotinamido)biphenyl-4-ylthio)pentanoate (**18**). A solution of aminobiphenyl **17** (100 mg, 0.28 mmol) and nicotinoyl chloride (**9**) (54 mg, 0.30 mmol) in anhydrous THF (1 mL) was stirred at rt for 3 h. After this time the solvent was removed under vacuum and the residue was purified by chromatography, eluting with hexane-Et_2O mixtures (from 8:2 to 2:8), to give amide **18** (120.3 mg, 87%) as an oil. IR ν_{max}/cm^{-1} (NaCl) 3394, 3284, 2976, 2932, 1725, 1675, 1580, 1521, 1398, 1367, 1154, 758; ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.44 (1H, d, *J* = 8.6 Hz, H-3' PhPh), 8.43 (1H, dd, *J* = 4.7, 1.9, H-6 Py), 8.22 (1H, br s, NH), 8.12 (1H, dd, *J* = 7.7, 1.9 Hz, H-4 Py), 7.5-7.2 (8H, m, H-2/H-6, H-3/H-5, H-4', H-5'. H-6' PhPh and H-5 Py), 2.96 (2H, t, *J* = 6.9 Hz, H-5), 2.24 (2H, t, *J* = 7.0 Hz, H-2), 1.72 (4H, m, H-3 and H-4), 1.43 (9H, s, CMe_3); ^{13}C NMR (75 MHz, $CDCl_3$) δ 172.62 (C-1), 162.45 (CON), 151.19 (C-6 Py), 146.72 (C-2 Py), 139.99 (C-4 Py), 137.45 (C-3 Py), 134.89, 134.40, 132.61, 131.18 (C-1, C-4, C-1', C-2' PhPh), 130.21 (C-4'), 129.84 and 128.85 (C-2/C-6 and C-3/C-5 PhPh), 128.54 (C-6' PhPh), 125.20, 122.81 and 121.74 (C-3', C-5' PhPh and C-5 Py), 80.19 (*C*Me_3), 34.95 (C-5), 32.82 (C-2), 28.40 (C-4), 28.06 (C*Me*$_3$), 24.21 (C-3); MS (EI) *m/z* 496 (M^+, 37), 433 (6), 442 (28), 441 (20), 440 (70), 423 (16), 379 (14), 340 (28), 140 (100); HRMS (EI), calcd for $C_{27}H_{29}{}^{35}ClN_2O_3S$ 496.15874, found 496.15887.

Preparation of *5-(2'-(2-chloronicotinamido)biphenyl-4-ylthio)pentanoic acid* (Hapten BL*c*). A solution of *tert*-butyl ester **18** (120.2 mg, 0.24 mmol) in HCOOH (5 mL) was stirred at rt for 1 h. The reaction mixture was concentrated under reduced pressure, $CHCl_3$ was added, and the mixture was concentrated again to leave a foam that was crystallized from benzene-hexane to afford hapten BL*c* (103 mg, 97%) as white crystals. Mp 117-118 ºC (from C_6H_6-hexane); IR ν_{max}/cm^{-1} (KBr) 3500-2500, 3304, 3023, 2919, 1704, 1659, 1580, 1519, 1446, 1401, 759; ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.64 (1H, br s, COOH), 8.44-8.41 (2H, m, H-6 Py and H-3' PhPh), 8.32 (1H, br s, NH), 8.16 (1H, dd, *J* = 7.7, 1.7 Hz, H-4 Py), 7.42-7.23 (8H, m, H-2/H-6, H-3/H-5, H-4', H-5'. H-6' PhPh and H-5 Py), 2.95 (2H, t, *J* = 6.6 Hz, H-5), 2.37 (2H, t, *J* = 6.5 Hz, H-2), 1.75 (4H, m, H-3 and H-4); ^{13}C NMR (75 MHz, $CDCl_3$) δ 179.20 (C-1), 162.21 (CON), 150.98 (C-6 Py), 146.46 (C-2 Py), 140.47 (C-4 Py), 136.95 (C-3 Py), 135.04, 134.35,

132.61, 131.16 (C-1, C-4, C-1', C-2' PhPh), 130.18 (C-4'), 129.89 and 129.16 (C-2/C-6 and C-3/C-5 PhPh), 128.53 (C-6' PhPh), 125.10, 122.96 and 121.65 (C-3', C-5' PhPh and C-5 Py), 34.95 (C-5), 32.79 (C-2), 28.44 (C-4), 23.98 (C-3); MS (EI) *m/z* 440 (M^+, 4), 178 (6), 167 (7), 161 (3), 149 (7), 140 (15), 83 (100); HRMS (EI), calcd for $C_{23}H_{21}{}^{35}ClN_2O_3S$ 440.09614, found 440.09557. UV (100 mM sodium phosphate buffer, pH 7.4) Є (280 nm) 10.72 mM−1 cm−1.

^{1}H NMR spectra of NHS esters of haptens BL*b* and BL*c*




NHS-ester hapten BLc
7.388
7.331
7.260 Chloroform-d
8.462
8.439
8.219
8.147
8.141
8.121
8.115
2.989
2.828
2.660
1.83
0.87
0.94
7.41
2.00
4.02
2.01
4.10
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
f1 (ppm)
NHS-ester of hapten BLc
(300 MHz, CDCl3)

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