Electronic Supplementary Information

Forchlorfenuron-Mimicking Haptens: from Immunogen Design to Antibody Characterization by Hierarchical Clustering Analysis

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1. General spectroscopic techniques

NMR spectra were recorded in $CDCl_3$ or DMSO-d_6 at room temperature 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 (7.26 and 2.50 ppm) and to solvent carbons in the ^{13}C NMR spectra (77.00 and 39.43 ppm). Carbon substitution degrees were established by distortionless enhancement by polarization transfer pulse sequences. A combination of correlation spectroscopy and heteronuclear single quantum coherence experiments was used for the assignment of ^{1}H and ^{13}C chemical shifts. IR spectra were measured as thin films between NaCl plates and KBr pellets for liquid and solid compounds, respectively, in a Nicolet Avatar 320 spectrometer. Electron-impact (EI) and fast atom bombardment (FAB) mass spectra (MS and HRMS) were carried out in a Micromass VG Autospec spectrometer. Extinction coefficients were calculated in 100 mM sodium phosphate buffer, pH 7.4 (PB).

2. Spectroscopic characterization data of haptens *m*2 and *s*3 and intermediates of their synthesis (Scheme 1 in the article)

2-(3-(3-(2-Chloropyridin-4-yl)ureido)phenyl)acetic acid (4, hapten *m*2)

^{1}H NMR (DMSO-*d*6), δ: 12.33 (1H, br s, OH), 9.32 and 8.99 (1H each, each s, two NH), 8.17 (1H, d, J = 5.9 Hz, H-6 Py), 7.66 (1H, d, J = 1.8 Hz, H-3 Py), 7.39 (1H, br s, H-2 Ph), 7.33 (1H, m partially overlapped with dd at 7.31, H-4 Ph), 7.31 (1H, dd, J = 5.7, 1.9 Hz, H-5 Py), 7.24 (1H, t, J = 7.7 Hz, H-5 Ph), 6.91 (1H, br d, J = 7.6 Hz, H-6 Ph), 3.53 (2H, s, H-2); ^{13}C NMR ($CDCl_3$/DMSO-*d*6), δ: 172.59 (C-1), 151.82 (NCON), 150.96 (C-4 Py), 149.93 (C-6 Py), 149.12 (C-2 Py), 138.74 (C-3 Ph), 135.68 (C-1 Ph), 128.72 (C-5 Ph), 123.79 (C-6 Ph), 119.56 (C-2 Ph), 117.05 (C-4 Ph), 111.88 (C-3 Py), 111.24 (C-5 Py), 40.80 (C-2); IR (KBr): 3400–2130, 1715, 1460–1620, 1375, 1183, 778, 723, 641, 443 cm^{-1}; MS (EI) *m/z*: 305 (M^+, 7.3), 287 (2), 261 (9), 177 (33),

154 (86), 151 (100); HRMS: calcd for $C_{14}H_{12}ClN_3O_3$ 305.05672, found 305.05541; UV (PB), ε (280 nm) = 11.35 $mM^{-1}cm^{-1}$, ε (260 nm) = 28.19 $mM^{-1}cm^{-1}$.

3-((4-(3-Phenylureido)pyridin-2-yl)thio)propanoic acid (11, hapten *s*3)

2-(3-Methoxy-3-oxopropylthio)-4-nitropyridine N-oxide (**7**):

^{1}H NMR ($CDCl_3$), δ: 8.31 (1H, d, *J* = 7.2 Hz, H-6 Py), 8.03 (1H, d, *J* = 3.0 Hz, H-3 Py), 7.89 (1H, dd, *J* = 7.2, 3.0 Hz, H-5 Py), 3.67 (3H, s, OCH_3), 3.28 (2H, t, *J* = 7.2 Hz, H-1), 2.82 (2H, t, *J* = 7.2 Hz, H-2); ^{13}C NMR ($CDCl_3$), δ: 170.98 (C-3), 153.88 (C-2 Py), 142.52 (C-4 Py), 139.08 (C-6 Py), 115.62 and 114.92 (C-3 and C-5 Py), 52.30 (OCH_3), 32.46 (C-2), 25.69 (C-1); IR (KBr): 3100, 3073, 3015, 2950, 1739, 1574, 1522, 1458, 1434, 1197, 1147, 873, 744, 651 cm^{-1}; MS (EI), *m/z*: 258 (M^+, 22), 242 (25), 241 (70), 227 (16), 225 (7), 211 (15), 209 (100), 199 (16), 183 (44), 182 (35), 172 (46), 163 (23),153 (24); HRMS: calcd for $C_9H_{10}N_2O_5S$ 258.03104, found 258.03076.

Methyl 3-((4-aminopyridin-2-yl)thio)propanoate (**8**):

^{1}H NMR ($CDCl_3$), δ: 8.04 (1H, d, *J* = 5.7 Hz, H-6 Py), 6.42 (1H, d, *J* = 2.3 Hz, H-3 Py), 6.27 (1H, dd, *J* = 5.7, 2.3 Hz, H-5 Py), 4.09 (2H, br s, NH_2), 3.69 (3H, s, OCH_3), 3.37 (2H, t, *J* = 7.2 Hz, H-3), 2.76 (2H, t, *J* = 7.2 Hz, H-2); ^{13}C NMR ($CDCl_3$), δ: 172.63 (C-1), 158.30 (C-2 Py), 152.62 (C-4 Py), 149.65 (C-6 Py), 107.15 (C-3 Py), 106.84 (C-5 Py), 51.72 (OCH_3), 34.72 (C-2), 24.94 (C-3); IR (film): 3465, 3375, 3210, 3005, 2950, 1734, 1636, 1592, 1550, 1479, 1437, 1360, 1258, 1132, 981, 823 cm^{-1}; MS (EI) *m/z*: 212 (M^+, 20), 181 (7), 179 (7), 155 (4), 154 (9), 153 (100), 152 (4), 139 (12), 127 (20), 126 (26); HRMS: calcd for $C_9H_{12}N_2O_2S$ 212.06195, found 212.06131.

Methyl 3-(4-((3-phenylureido)pyridin-2-y)lthio)propanoate (**10**):

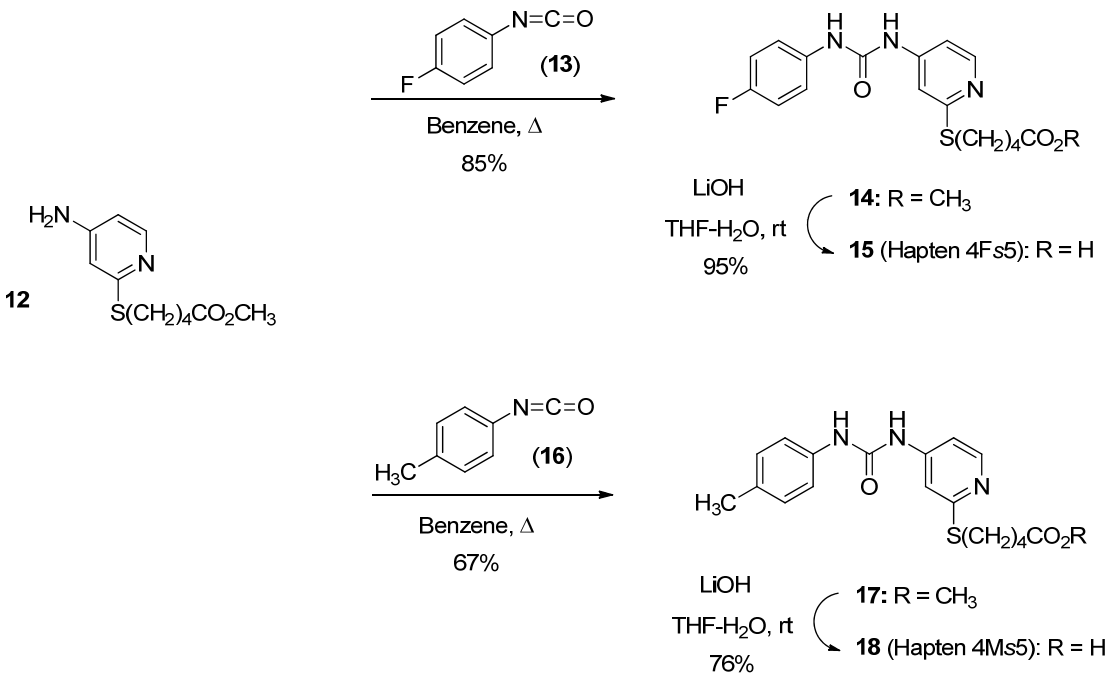
^{1}H NMR ($CDCl_3$), δ: 8.10 (1H, d, *J* = 6.0 Hz, H-6 Py), 7.90 and 7.74 (1H each, each br s, two NH), 7.30–7.20 (4H, m, H-2/H-6 and H-3/H-5 Ph), 7.19 (1H, d, *J* = 1.9 Hz, H-3 Py), 7.05 (1H, m, H-4 Ph), 6.95 (1H, dd, *J* = 6.0, 1.9 Hz, H-5 Py), 3.67 (3H, s, OCH_3), 3.35 (2H, t, *J* = 7.2 Hz, H-3), 2.75 (2H, t, *J* = 7.2 Hz, H-2); ^{13}C NMR ($CDCl_3$), δ: 173.24 (C-1), 158.80 (C-2 Py), 152.83 (NCON), 149.72 (C-6 Py), 146.24 (C-4 Py), 137.36 (C-1 Ph), 129.22 (C-3/C-5 Ph), 124.47 (C-4 Ph), 120.88 (C-2/C-6 Ph), 110.57 (C-3 Py), 110.04 (C-5 Py), 52.01 (OCH_3), 34.50 (C-2), 25.23 (C-3); IR (KBr): 3392, 3354, 2950, 1722, 1698, 1579, 1540, 1439, 1275, 1200, 1083, 1002, 753 cm^{-1}; MS (EI) *m/z*:

331 (M^+, 5), 330 (1), 298 (1), 273 (2), 272 (13), 238 (14), 212 (8), 179 (82), 153 (36), 119 (45), 93 (100); HRMS: calcd for $C_{16}H_{17}N_3O_3S$ 331.09906, found 331.09777.

3-((4-(3-Phenylureido)pyridin-2-yl)thio)propanoic acid (**11**, hapten *s*3):

^{1}H NMR (DMSO-*d*6), δ: 12.34 (1H, br s, OH), 9.52 and 9.27 (1H each, each br s, two NH), 8.21 (1H, d, *J* = 5.7 Hz, H-6 Py), 7.50–7.45 (3H, m, H-2/H-6 Ph and H-3 Py), 7.28 (2H, t, *J* = 7.9 Hz, H-3/H-5 Ph), 7.11 (1H, dd, *J* = 5.7, 1.9 Hz, H-5 Py), 6.99 (1H, tt, *J* = 7.9, 1.2 Hz, H-4 Ph), 3.27 (2H, t, *J* = 7.0 Hz, H-5), 2.60 (1H, *J* = 7.0 Hz, H-2); ^{13}C NMR (DMSO-*d*6), δ: 173.50 (C-1), 158.28 (C-2 Py), 152.14 (NCON), 149.59 (C-6 Py), 146.93 (C-4 Py), 139.13 (C-1 Ph), 128.73 (C-3/C-5 Ph), 122.26 (C-4 Ph), 118.49 (C-2/C-6 Ph), 109.25 (C-3 Py), 108.75 (C-5 Py), 34.62 (C-2), 24.83 (C-3); IR (KBr): 3550–2400, 3306, 2922, 1731, 1599, 1557, 1189, 1083, 751 cm^{-1}; MS (EI) *m/z*: 317 (M^+, 0.5), 290 (0.6), 273 (0.8), 245 (1.5), 224 (1.2), 198 (5), 180 (10), 153 (17), 126 (67), 119 (71), 93 (100); HRMS (FAB): calcd for $C_{15}H_{16}N_3O_3S$ [M^++1] 318.09124, found 318.09063; UV (PB), ε (280 nm) = 11.31 $mM^{-1}cm^{-1}$, ε (260 nm) = 29.88 $mM^{-1}cm^{-1}$.

3. Preparation of haptens 4F*s*5 and 4M*s*5



Scheme S1. Preparation of haptens 4F*s*5 and 4M*s*5

5-(4-(3-((4-Fluorophenyl)ureido)pyridin-2-yl)thio)pentanoic acid (15, hapten 4F*s*5)

Methyl 5-((4-(3-(4-fluorophenyl)ureido)pyridin-2-yl)thio)pentanoate (**14**). Methyl 5-((4-aminopyridin-2-yl)thio)pentanoate (**12**) was synthesized as described previously for the preparation of hapten *s*5.[1] Then, 4-fluorophenyl isocyanate (**13**, 29 μL, 0.26 mmol) was added drop wise to a solution of the amino ester **12** (57 mg, 0.24 mmol) in benzene (1.0 mL). After 1 h 45 min at reflux under argon, the reaction mixture was diluted with hexane and the solid was filtrated affording the urea-methyl ester **14** (76 mg, 85%) as a white solid: mp 154–158 ºC (from acetone). ^{1}H NMR (acetone-*d*6), δ: 8.43 and 8.31 (1H each, two s, two NH), 8.19 (1H, d, *J* = 5.7 Hz, H-6 Py), 7.56–7.52 (3H, m, H-3 Py and H-2/H-6 Ph), 7.12 (1H, dd, *J* = 5.7, 1.8 Hz, H-5 Py), 7.10–7.04 (2H, m, H-3/H-5 Ph), 3.61 (3H, s, CO_2CH_3), 3.17 (2H, t, *J* = 6.6 Hz, H-5), 2.37 (2H, t, *J* = 7.2 Hz, H-2), 1.73 (4H, m, H-3 and H-4); ^{13}C NMR (acetone-*d*6), δ: 174.91(C-1), 161.42 (NCON), 160.40 (d, *J* = 239 Hz, C-4 Ph), 153.89 (C-2 Py), 151.48 (C-6 Py), 148.76 (C-4 Py), 135.60 (d, *J* = 2.1 Hz, C-1 Ph), 122.69 (d, *J* = 7.5 Hz, C-2/C-6 Ph), 117.10 (d, *J* = 22 Hz, C-3/C-5 Ph), 111.25 and 111.11 (H-3 and H-5 Py), 52.50 (CO_2CH_3), 34.85 (C-5), 30.91 (C-2), 30.74 (C-4), 25.83 (C-3); IR (KBr): 3360, 3131, 3071, 3044, 2956, 2933, 2874, 1694, 1585, 1541, 1508, 1121, 837, 793 cm^{-1}; MS (EI) *m/z*: 277 (2.5), 290 (3), 277 (7.5), 267 (3), 266 (19), 263 (3), 240 (5), 236 (2.5), 235 (18), 233 (59), 219 (5), 209 (6), 179 (82), 166 (86), 152 (64), 120 (20), 19 (22), 111 (100); HRMS (EI): calcd for $C_{18}H_{20}FN_3O_3S$ [M^+] 377.12094, found 377.12086.

5-(4-(3-((4-Fluorophenyl)ureido)pyridin-2-yl)thio)pentanoic acid (**15**, hapten 4F*s*5). Hapten 4F*s*5 was obtained by hydrolysis of the methyl ester moiety of compound **14** (62 mg, 0.16 mmol) with $LiOH·H_2O$ (69 mg, 1.65 mmol) during 6 h, as described for hapten *s*3 (see Experimental section in the article). After ethyl ether extraction and acidification of the aqueous layer with $KHSO_4$, followed by extraction with AcOEt and work up afforded nearly pure hapten 4F*s*5 (**15**, 57 mg, 95%) as a white solid: mp 197–199 ºC (from DMSO/H_2O). ^{1}H NMR (DMSO-*d*6), δ: 12.03 (1H, broad s, OH), 9.21 y 9.24 (1H each, two s, two NH), 8.20 (1H, d, *J* = 5.7 Hz, H-6 Py), 7.49–7.43 (3H, m, H-3 Py

[1] C. Suárez-Pantaleón, J.V. Mercader, C. Agulló, A. Abad-Somovilla and A. Abad-Fuentes, *J. Agric. Food Chem.* 2010, **58**, 8502–8511.

and H-2 and H-6 Ph), 7.07 (1H, dd, *J* = 5.7, 1.9 Hz, H-5 Py), 7.13 (2H, m, H-3 and H-5 Ph), 3.10 (2H, t, *J* = 6.6 Hz, H-5), 2.24 (2H, t, *J* = 6.6 Hz, H-2), 1.63 (4H, m, H-3 and H-4); ^{13}C NMR (DMSO-*d*6), δ: 174.4(C-1), 158.74 (NCON), 157.60 (d, *J* = 239 Hz, C-4 Ph), 152.13 (C-2 Py), 149.57 (C-6 Py), 146.74 (C-4 Py), 135.34 (d, *J* = 2.3 Hz, C-1 Ph), 120.40 (d, *J* = 7.7 Hz, C-2/C-6 Ph), 115.28 (d, *J* = 22 Hz, C-3 and C-5 Ph), 109.16 and 108.75 (H-3 and H-5 Py), 33.24 (C-5), 28.76 and 28.54 (C-2 and C-4), 23.73 (C-3); IR (KBr): 3340–3276, 3153, 3073, 2956, 2934, 2918, 2718, 1565, 1509, 1192, 830, 740 cm^{-1}; MS (EI) *m/z*: 207 (1), 138 (5), 137 (70), 112 (4), 111 (53), 110 (6), 108 (4), 73 (100); HRMS (FAB): calcd for $C_{17}H_{19}FN_3O_3S$ [M^+ + 1] 364.11312, found 364.11276; UV (PB), ε (280 nm) = 8.00 mM^{-1}cm^{-1}, ε (260 nm) = 25.44 mM^{-1}cm^{-1}.

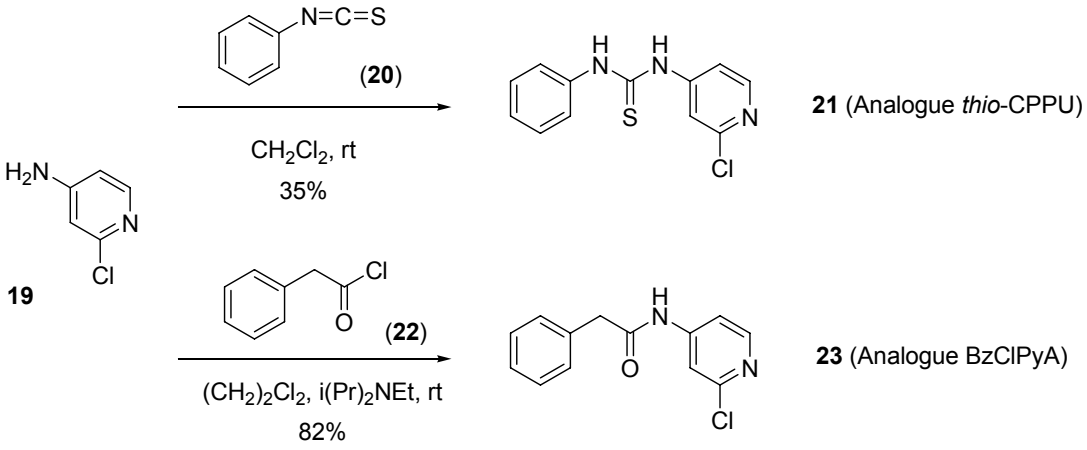
5-(4-((3-p-Tolylureido)pyridin-2-yl)thio)pentanoic acid (18, hapten 4M*s*5)

Methyl 5-((4-(3-p-tolylureido)pyridin-2-yl)thio)pentanoate (**17**). *p*-Tolyl isocyanate (**16**, 32 μL, 0.25 mmol) was added drop wise to a solution of methyl 5-((4-aminopyridin-2-yl)thio)pentanoate (**12**, 55 mg, 0.23 mmol) in dry benzene (1.1 mL) under inert atmosphere. The reaction was stirred for 1 h 45 min at reflux and then it was diluted with hexane and filtrated to afford the corresponding urea-methyl ester **17** (57 mg, 67%) as a yellowish solid: mp 136–140 ºC (from acetone). ^{1}H NMR (acetone-*d*6), δ: 8.37 and 8.17 (1H each, two s, two NH), 8.19 (1H, d, *J* = 5.8 Hz, H-6 Py), 7.54 (1H, d, *J* = 1.6 Hz, H-3 Py), 7.40 (2H, m, H-2 and H-6 Ph), 7.13–7.06 (3H, *m*, H-5 Py and H-3 and H-5 Ph), 3.61 (3H, s, CO_2CH_3), 3.17 (2H, t, *J* = 7.0 Hz, H-5), 2.35 (2H, t, *J* = 7.0 Hz, H-2), 2.27 (3H, s, CH_3-Ph), 1.73 (4H, m, H-3 and H-4); ^{13}C NMR (acetone-*d*6), δ 174.91(C-1), 161.36 (NCON), 153.81 (C-2 Ph), 151.44 (C-6 Py), 148.88 (C-4 Py), 138.53 (C-4 Ph), 133.91 (C-1 Ph), 131.07 (C-3 and C-5 Ph), 120.94 (C-2 and C-6 Ph), 111.17 and 111.06 (H-3 and H-5 Py), 52.50 (CO_2CH_3), 34.85 (C-5), 30.92 (C-2), 30.74 (C-4), 25.83 (C-3), 21.72 (CH_3-Ph); IR (KBr): 3364, 1698, 1595, 1535, 1480, 1362, 1168, 821, 630 cm^{-1}; MS (EI) *m/z*: 373 (3), 286 (3), 273 (10), 266 (18), 259 (4), 240 (7), 235 (18), 233 (4), 219 (5), 179 (75), 166 (77), 165 (19), 153 (28), 106 (100); HRMS (EI): calcd for $C_{19}H_{23}N_3O_3S$ [M^+] 373.14601, found 373.14703.

5-((4-(3-p-Tolylureido)pyridin-2-yl)thio)pentanoic acid (**18**, hapten 4M*s*5). The hydrolysis of the ester moiety of **17** (57 mg, 0.15 mmol) was carried out essentially as described for haptens *s*3 and

4F*s*5, using LiOH·H_2O (65 mg, 1.54 mmol). After ethyl ether extraction and acidification of the aqueous layer, the precipitated solid was filtered and dried under vacuum to give pure hapten 4M*s*5 (**18**, 42 mg, 76%) as a white solid: mp 198–201 ºC (from MeOH). ^{1}H NMR (DMSO-*d*6), δ: 12.02 (1H, broad s, OH), 9.06 and 8.79 (1H each, two s, two NH), 8.19 (1H, d, *J* = 5.6 Hz, H-6 Py), 7.43 (1H, d, *J* = 1.7 Hz, H-3 Py), 7.33 (2H, m, H-2 and H-6 Ph), 7.10 (2H, *m*, H-3 and H-5 Ph), 7.06 (1H, dd, *J* = 5.7, 1.7 Hz, H-5 Py), 3.10 (2H, t, *J* = 7.0 Hz, H-5), 2.24 (2H, t, *J* = 7.3 Hz, H-2), 1.63 (4H, m, H-3 and H-4); ^{13}C NMR (DMSO-*d*6), δ: 174.26(C-1), 158.71 (NCON), 151.98 (C-2 Ph), 149.55 (C-6 Py), 146.75 (C-4 Py), 136.35 (C-4 Ph), 131.28 (C-1 Ph), 129.15 (C-3 and C-5 Ph), 118.60 (C-2 and C-6 Ph), 109.10 and 108.66 (H-3 and H-5 Py), 33.13 (C-5), 28.74 and 28.53 (C-2 and C-4), 23.67 (C-3), 20.29 (CH_3-Ph); IR (KBr): 3115, 3049, 3022, 2945, 1720, 1606, 1560, 1508, 1406, 1315, 1204, 841, 818 cm^{-1}; MS (EI) *m/z*: 133 (10), 132 (5), 108 (1), 107 (14), 106 (18), 105 (1.5), 91 (2), 73 (100); HRMS (FAB): calcd for $C_{18}H_{22}N_3O_3S$ [M^+ + 1] 360.13819, found 360.13877; UV (PB), ε (280 nm) = 14.51 mM^{-1}cm^{-1}, ε (260 nm) = 30.75 mM^{-1}cm^{-1}.

4. Preparation of CPPU analogues *thio*-CPPU and BzClPyA



Scheme S2. Preparation of CPPU analogues *thio*-CPPU and BzClPyA

1-(2-Chloro-4-pyridyl)-3-phenylthiourea (21, *thio*-CPPU)

Phenyl isothiocyanate (**20**, 354 µL, 1.86 mmol) was added drop wise via syringe to a solution of 4-amino-2-chloropyridine (**19**, 217 mg, 1.69 mmol)[2] in dry CH_2Cl_2 (2.0 mL) and then stirred at rt for 120 h. The reaction mixture was then extracted with CH_2Cl_2, washed with water and brine, and dried over Mg_2SO_4 to give the crude product that was purified by column chromatography, using CH_3Cl/MeOH 9:1 as eluent. The eluted fraction containing the product was extracted again with AcOEt and washed with 1M HCl. The organic layer was then concentrated and dried affording *thio*-CPPU (**21**, 154 mg, 35%) as a yellow solid; mp 135–138 ºC (from MeOH). ^{1}H NMR (DMSO-*d*6), δ: 10.40 (1H, s, NH), 10.37 (1H, s, NH), 8.24 (1H, d, *J* = 5.6 Hz, H-6 Py), 7.87 (1H, d, *J* = 1.8 Hz, H-3 Py), 7.54 (1H, dd, *J* = 5.6, 1.8 Hz, H-5 Py), 7.49 (2H, dt, *J* = 7.5, 1.2 Hz, H-2/H-6 Ph), 7.39 (2H, t, *J* = 7.5, Hz, H-3/H-5 Ph), 7.19 (1H, tt, *J* = 7,5, 1,2Hz, H-4 Ph); ^{13}C NMR (DMSO-*d*6), δ: 179.08 (NCON), 150.25 (C-4 Py), 149.72 (C-6 Py), 149.41 (C-2 Py), 138.62 (C-1 Ph), 128.61 (C-2/C-6 Ph), 125.19 (C-4 Ph), 123.79 (C-3/C-5 Ph), 114.62 and 114.15 (C-3 and C-5 Py); IR (KBr): 3152, 3115, 2968, 1589, 1569, 1528, 1488, 1190, 749 cm^{-1}; MS (EI) *m/z*: 263 (8), 262 (2), 230 (15), 229 (100), 195 829, 194 (16), 178 (2), 173 (20), 170 (50); HRMS (EI): calcd for $C_{12}H_{10}ClN_3S$ 263.02839, found 263.02904.

***N*-(2-Chloro-4-pyridyl)-2-phenylacetamide (23, BzClPyA)**

2-phenylacetyl chloride (**22**, 202 µL, 1.52 mmol) was added to a solution containing 4-amino-2-chloropyridine (**19**, 98 mg, 0.76 mmol) and *N*,*N*'-diisopropil ethylamine (i(Pr)$_2$NEt, 290 µL, 1.68 mmol) in anhydrous 1,2-dichloroethane (2.0 mL). The mixture was stirred at rt for 1.5 h under inert atmosphere, then the solvent was eliminated and the crude product was diluted with water and extracted three times with EtOAc. The combined organic layers were washed with brine, dried over Na_2SO_4, and concentrated under vacuum to obtained an oil that was purified by column chromatography, using CH_3Cl as eluent, to afford pure BzClPyA (**23**, 155 mg, 82%) as a slightly colored oil. ^{1}H NMR ($CDCl_3$), δ: 8.70 (1H, s, NH), 8.12 (1H, d, *J* = 5.6 Hz, H-6 Py), 7.59 (1H, d *J* =

[2] S. Kasmi-Mir, A. Djafri, L. Paquin, J. Hamelin and M. Rahmouni, *Molecules,* 2006, **11**, 597–602.

1.6 Hz, H-3 Py), 7.34–7.21 (6H, m, H-5 Py and H-2/H-3/H-4/H-5/H-6 Ph), 3.67 (CH_2O); ^{13}C NMR ($CDCl_3$), δ: 170.46 (CON), 152.12 (C-2-Py), 149.79 (C-6 Py), 147.34 (C-4 Py), 133.41 (C-1 Ph), 129.17 (C-2/C-6 Ph), 129.04 (C-3/C-5 Ph), 127.69 (C-4 Ph), 113.56 and 112.58 (C-3 and C-5 Py), 44.38 (CH_2O); IR (KBr): 3263, 3161, 3062, 3029, 1685, 1581, 1509, 1371, 1266, 1159, 1126,1079, 837, 731, 718, 694 cm^{-1}; MS (EI) *m/z*: 246 (31), 131 (2), 129 (8), 119 (3), 118 (24), 92 (29), 91 (100); HRMS (EI): calcd for $C_{13}H_{11}ClN_2O$ 246.05599, found 246.05649.

5. Preparation of protein conjugates

Immunizing conjugate. The active ester method was used to couple haptens *m*2 and *s*3 to bovine serum albumin (BSA). Typically, a 100 mM hapten solution was prepared in *N*,*N*-dimethylformamide (DMF) and mixed with 1 molar equivalent of *N*-hydroxysuccinimide and 1 molar equivalent of *N*,*N*'-dicyclohexylcarbodiimide also in DMF. Additional DMF was added to bring the final concentration of all reagents to 50 mM. The hapten was activated overnight at rt in amber vials. The day after, the reaction was centrifuged and the supernatant was collected. Next, 400 µL of activated *m*2 or *s*3 hapten solution was added drop wise to 2.0 mL of a 15 mg/mL BSA solution in 50 mM sodium carbonate–bicarbonate buffer, pH 9.6 (CB). The coupling reaction was incubated during 4 h at rt with moderate stirring. The initial hapten-to-protein molar ratio (MR) in the mixture was approximately 44:1. Finally, the conjugate was separated from uncoupled hapten by gel filtration on Sephadex G-25, using 100 mM sodium phosphate buffer, pH 4.4 (PB) as eluent. The degree of hapten-to-protein conjugation was measured spectrophotometrically. If conjugation occurred, the UV–vis spectrum of the conjugate was slightly different from that of the free protein. Therefore, the final average hapten-to-protein MR was calculated from the absorbance value at 280 nm by assuming that the molar absorption of the hapten and the protein were the same for the free and the conjugated forms. The purified conjugate was diluted to 1.0 mg/mL with PB and stored at −20 ºC. The calculated final hapten-to-protein MR are listed in Table S1.

Coating conjugates. Haptens *m*2, *s*3, 4F*s*5, and 4M*s*5 were linked to ovalbumin (OVA) by the mixed anhydride method. Typically, 200 µL of a solution with the hapten, tributylamine, and isobutyl chloroformate in DMF were prepared. The concentration of the reagents in the solution was 90 mM. After 1 h at rt with gentle stirring, 100 µL of the solution containing the activated hapten was added drop wise to 2.0 mL of a 15 mg/mL solution of OVA in CB. In this case, the initial hapten-to-protein MR was approximately 13:1. The coupling reaction was incubated during 2.5 h in the same conditions as described before and then the conjugates were purified following the same

procedure. MRs were determined by UV spectrophotometry. A solution of each conjugate was prepared at a concentration of 1.0 mg/mL in PB containing 0.01% (w/v) thimerosal, and it was stored at −20 ºC for daily usage. The final MRs can be found in Table S1. Hapten *s*3 was also coupled to OVA by the active ester method using an initial hapten-to-protein MR of 15:1.

Enzyme tracers. A 1/10 dilution of the activation mixture in DMF, prepared for the previous conjugates, was employed containing 0.5 μmol of the activated hapten. This dilution was added to 1.0 mL of a solution of horseradish peroxidase (HRP) in CB at a concentration of 2.2 mg/mL. The initial hapten-to-protein MR in the mixture was 10:1. The conjugation was incubated during 4 h and then the enzyme tracer was separated from the uncoupled hapten as described before. The final MR was calculated using, in this case, the absorbance values obtained at 400 nm and 280 nm. A working solution of the enzyme tracer was prepared in PBS containing 1% (w/v) BSA and 0.01% (w/v) thimerosal at a concentration of 0.5 mg/mL and was stored for its daily usage at 4 ºC. The remaining fraction was stored in elution buffer at −20 ºC. For the final MRs see Table S1. Hapten *s*3 was also coupled to HRP by the active ester method using an initial hapten-to-protein MR of 10:1.

Table S1. Hapten-to-protein MRs of conjugates

	Carrier protein		
Hapten	BSA	OVA	HRP
*p*2	24	2.8	2.1
*p*6	30	2.4	3.4
CldPhU*p*6	-[a]	4.5	7.5
PhPyU*p*6	-	2.5	1.5
*m*2	34	1.6	1.3
*m*6	34	3.0	3.2
*s*3	26	4.0[b]	1.3[b]
*s*5	18	6.4	3.3
4F*s*5	-	5.5	3.5
4M*s*5	-	5.9	3.8

[a] Not prepared. [b] These assay conjugates were prepared by the active ester method.

6. Monoclonal antibody production

Immunization and cell fusion. Female BALB/c mice (8–10 weeks old) were immunized by intraperitoneal injections with BSA–*m*2, BSA–*m*6, BSA–*s*3, and BSA–*s*5 conjugates (typically 4 animals per immunogen). Initially, animals received a first dose of immunogen consisting of 100 μg of conjugate in PB as a 1:1 emulsion with complete Freund's adjuvant (200 μL per mouse). Mice received two additional boosts at intervals of three weeks with the same amount of immunogen emulsified, in this case, with incomplete Freund's adjuvant. Finally, four days before cell fusions, every mouse received a last immunization containing 100 μg of conjugate diluted in sterile PBS. Cell fusions were carried out following standard chemical fusion procedures[3] using polyethylene glycol as fusing agent at a cellular ratio lymphocyte:myeloma of 4:1. The fused cells were cultured in 96-well culture plates at a density between 1.5×10^5 and 2.5×10^5 cells per well in DMEM supplemented with 15% (v/v) fetal bovine serum.

Hybridoma selection. Approximately 10 to 12 days after the fusions, the supernatants of the cultured plates were evaluated in order to identify antibody-secreting hybridomas with the ability to recognize both the coating conjugate and the free analyte. Typically, ELISA plates were coated with the homologous conjugate at a concentration of 1 μg/mL. The supernatants were evaluated simultaneously in adjacent ELISA wells: one as blank and the other with 1 μM forchlorfenuron (CPPU). Those clone-containing wells affording signal intensities in the absence of analyte close to saturation and more than 50% inhibition under competitive conditions were selected and further cloned by limiting dilution. This process was repeated as many times as necessary in order to guarantee the stability and the monoclonal character of the cell lines. Few positive signals were observed in the screening assays of cell fusions from *s*3 immunized mice. Therefore, two additional mice (*s*3#5 and *s*3#6) were immunized with the same conjugate but the cell-fusion screening assays were performed with the OVA–*s*5 conjugate. Using this strategy it was possible to stabilize 5 additional hybridomas (Table S2).

[3] J.V. Mercader and A. Montoya, *J. Agric. Food Chem.,* 1999, **47**, 1276–1284.

Monoclonal antibody purification. Immunoglobulins were purified from late stationary phase culture supernatants by ammonium sulfate precipitation followed by affinity chromatography following the manufacturer's instructions. Stock solutions of every antibody were prepared in PBS containing 1% (w/v) BSA and 0.01% (w/v) thimerosal and they were stored at 4 ºC for daily usage. The remaining volume was 2-fold precipitated with a saturated ammonium sulfate solution for its long-term conservation and it was stored at 4 ºC.

Table S2. Summary of cell fusions performed with mice immunized with conjugates BSA–*m*2, BSA–*m*6, BSA–*s*3, and BSA–*s*5

Immunized mouse	Growth yield[a]	Wells			Stabilized hybridoma
		Cultured	Positive[b]	Competitive[c]	
*m*2#1	74	384	11	5	4
*m*2#2	74	480	345	304	0
*m*2#3	---[d]				
*m*2#4	---				
*m*6#1	42	384	13	10	1
*m*6#2	---				
*m*6#3	67	384	9	1	1
*m*6#4	73	288	210	195	2
*s*3#1[e]	79	288	0	0	0
*s*3#2[e]	49	288	225	171	1
*s*3#3[e]	54	240	0	0	0
*s*3#4[e]	21	288	3	2	0
*s*3#5[f]	76	576	20	9	4
*s*3#6[f]	62	672	10	2	1
*s*5#1	77	384	89	77	2
*s*5#2	85	384	56	43	1
*s*5#3	77	672	491	472	4
*s*5#4	17	192	0	0	0

[a] Percentage of cultured wells with cellular growth by visual inspection at day 11 after fusion. [b] Wells with cellular growth affording $A_{492} \geq 1$ in the absence of analyte. [c] Wells with an inhibition of the signal intensity ≥ 50% in the presence of 1 µM CPPU. [d] These mice died during the immunization process. [e] The screening assay was performed with the conjugate OVA–*s*3 prepared by the mixed anhydride method. [f] The screening was performed with the heterologous conjugate OVA–*s*5.

7. Standard curve parameters

Table S3. Curve parameters of the selected cELISAs

i-cELISA						
mAb	OVA conjugate	[OVA conj.] (μg/mL)	[mAb] (ng/mL)	A_{max}	slope	IC_{50} (nM)
*p*6#24	4M*s*5	1.0	30	1.25 ± 0.02	−1.24 ± 0.13	0.16 ± 0.01
*m*2#15	*m*2	0.1	30	1.06 ± 0.09	−1.31 ± 0.13	0.22 ± 0.04
*s*3#51	*p*2	1.0	30	1.26 ± 0.07	−1.23 ± 0.07	0.08 ± 0.01
d-cELISA						
mAb	Enzyme tracer	[Tracer] (ng/mL)	[mAb] (μg/mL)	A_{max}	slope	IC_{50} (nM)
*p*2#61	*m*6	10	1.0	1.23 ± 0.03	−1.13 ± 0.09	0.23 ± 0.01
*m*2#15	*m*6	3	1.0	0.94 ± 0.07	−1.08 ± 0.10	0.32 ± 0.04
*s*5#34	*p*6	10	1.0	0.76 ± 0.07	−1.36 ± 0.06	0.21 ± 0.04

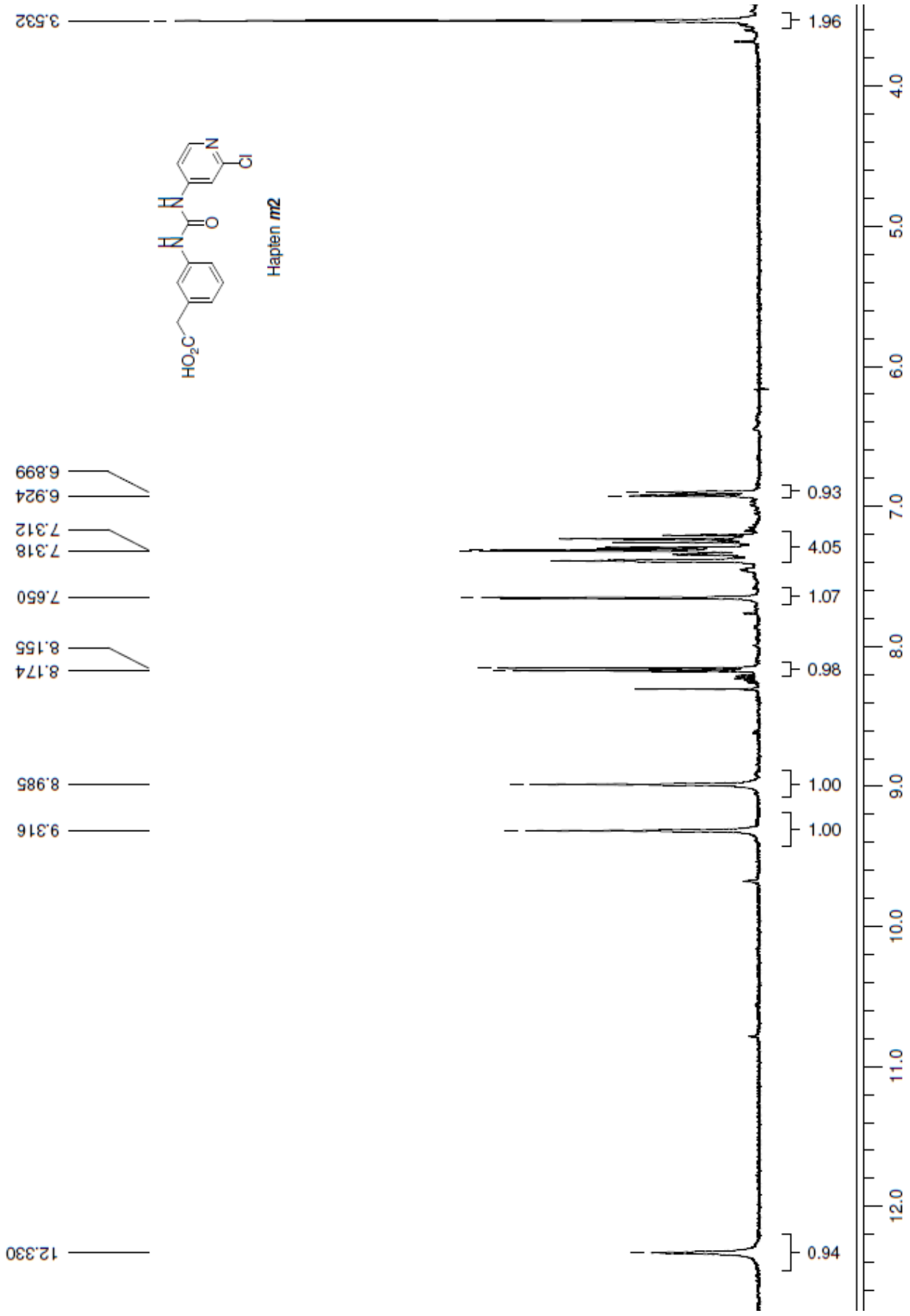
8. Antibody-distance data

Table S4. Minimum and maximum Kendall's tau correlation coefficient (τ) between antibodies by comparison of pairs of clusters[a]

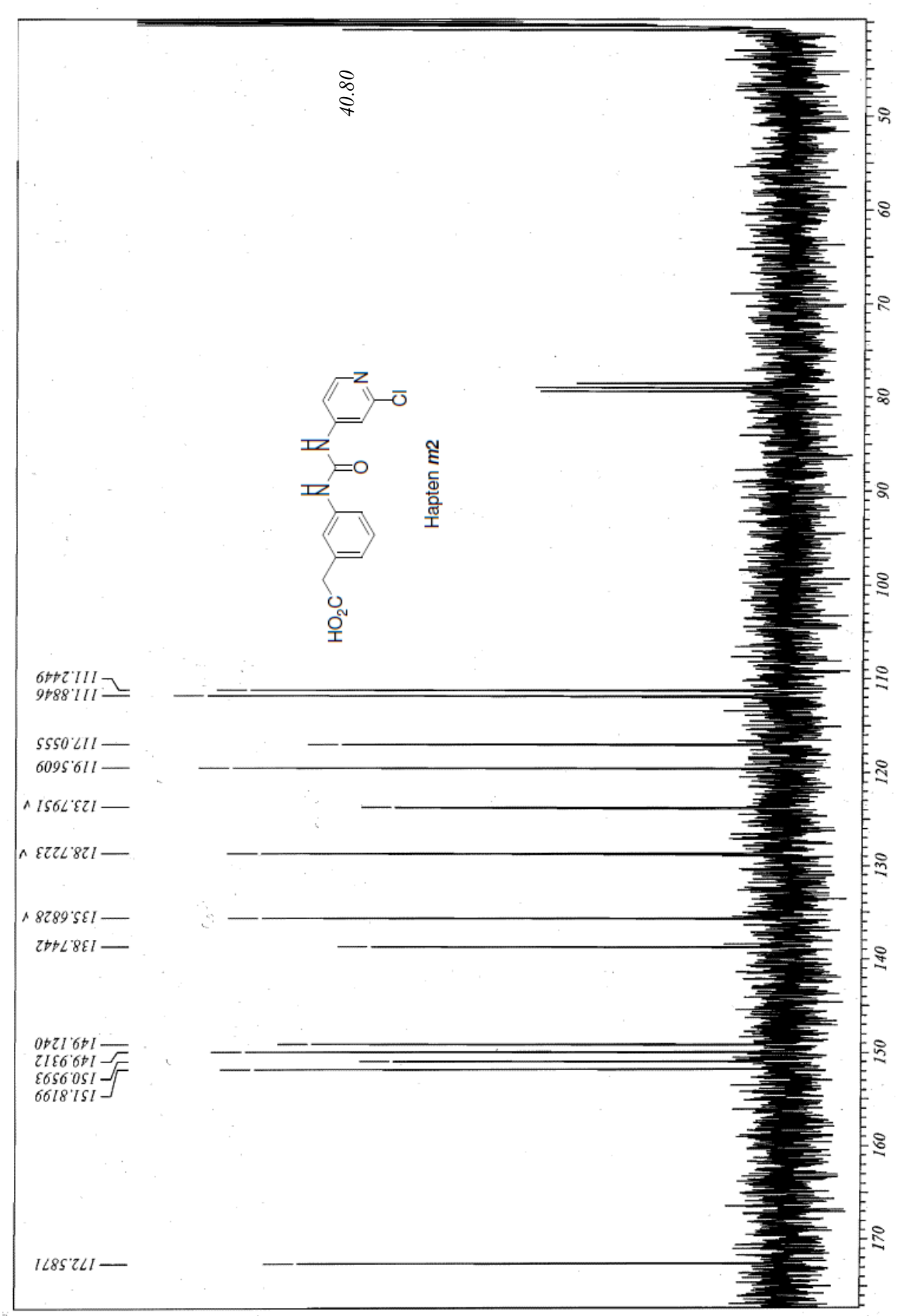
Confronted clusters	Lowest similarity		Highest similarity	
	τ[b]	Representative pairs	τ	Representative pairs
A1 vs A1	**0.41**	***m*6#43 vs *m*2#13**	**0.96**	***p*2#63 vs *p*2#62**
A1 vs A2	0.16	*m*6#43 vs *p*6#33	0.82	*m*2#12 vs *p*2#65/*m*2#14
A1 vs B1	−0.21	*m*6#43 vs *s*5#11	0.38	R*m*6#2 vs R*s*5#1
A1 vs B2	−0.25	*m*6#43 vs *m*6#13	0.51	R*m*6#1 vs *s*5#21
A2 vs A2	**0.60**	***p*6#42 vs *p*6#33**	**0.98**	***m*2#15 vs *p*2#65; *m*6#31 vs *p*2#65/*m*2#14**
A2 vs B1	−0.18	*p*2#66 vs *s*5#32	0.49	*p*6#21 vs R*s*5#1/*s*5#35; *p*6#33 vs R*s*5#1
A2 vs B2	−0.08	*p*6#42 vs *s*3#22	0.60	*p*6#33 vs *s*5#21
B1 vs B1	**0.01**	***p*6#41 vs *s*5#32**	**0.80**	**R*s*5#1 vs R*s*5#2**
B1 vs B2	−0.05	*s*5#35 vs *s*3#22	0.76	R*s*5#2 vs *s*3#52
B2 vs B2	**0.21**	***s*3#22 vs *s*3#52/*s*5#34/*s*3#53**	**0.91**	***s*3#52 vs *s*5#34**

[a] The combinations for antibodies inside the same cluster are indicated in bold-type letter. [b] τ ranges from −1 to 1, indicating the lowest and highest similarity, respectively.

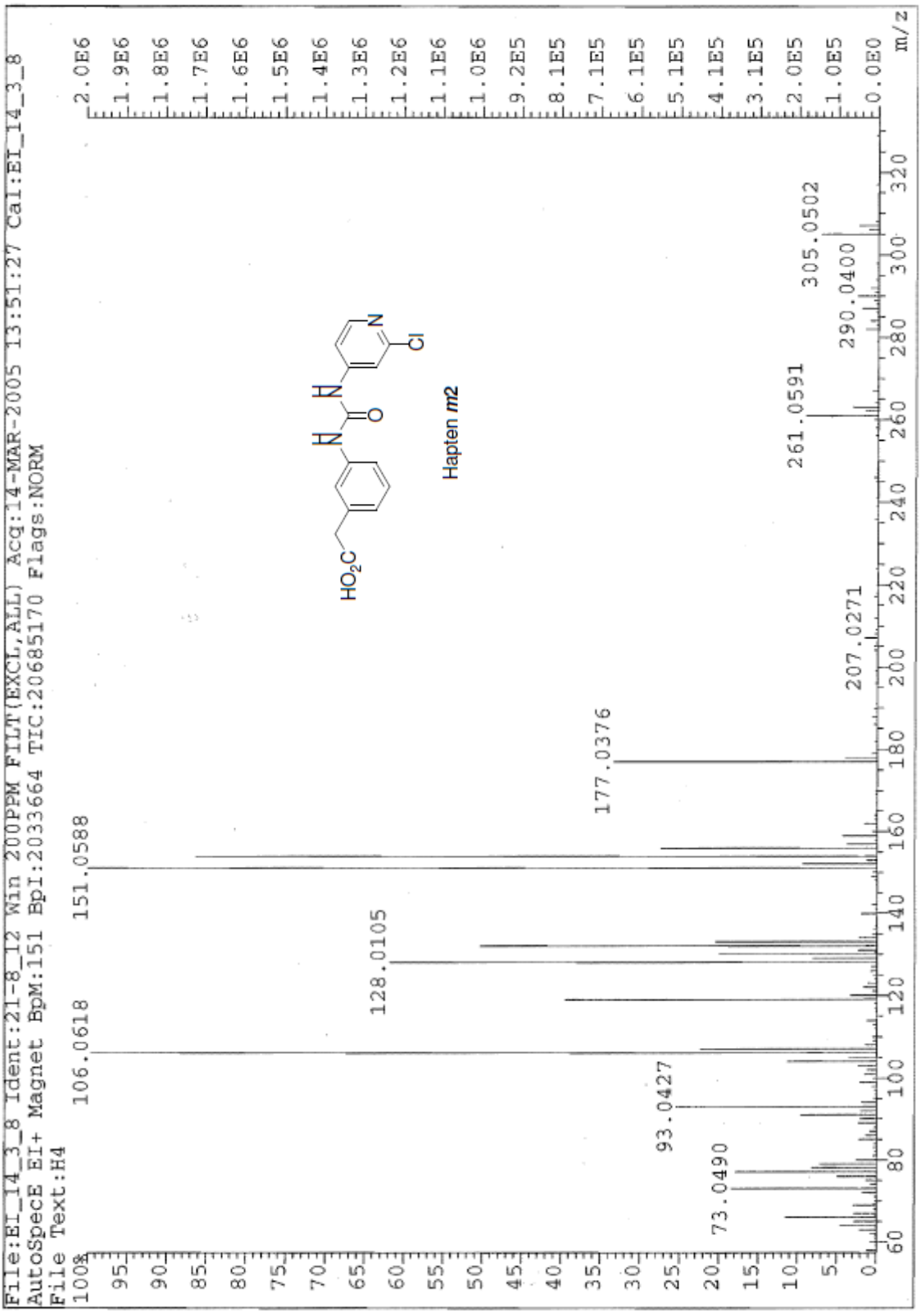
9. Copies of ^{1}H NMR, ^{13}C NMR, and mass spectra of haptens *m*2, *s*2, 4F*s*5, and 4M*s*5



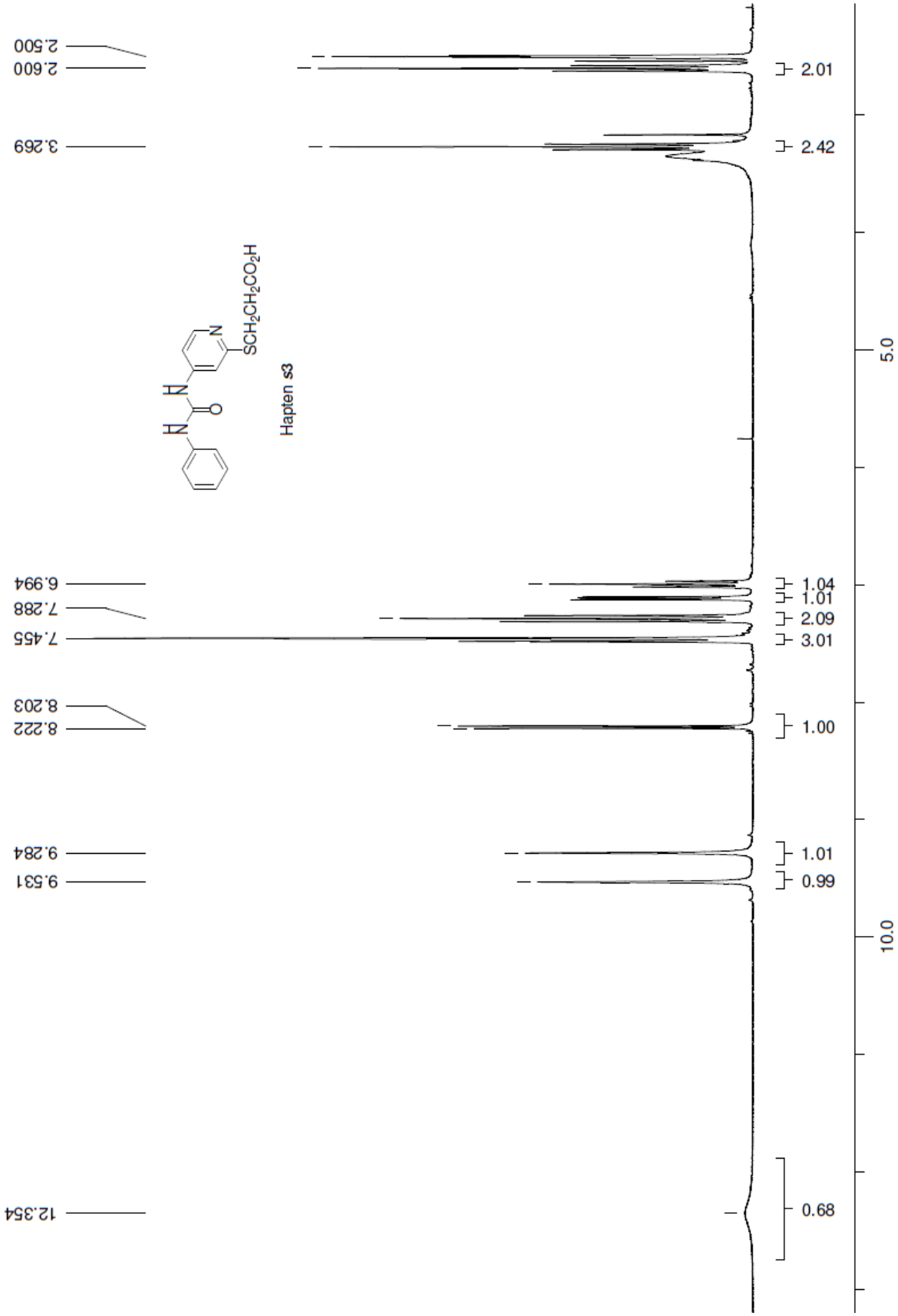
^{1}H NMR spectrum of 2-(3-(3-(2-chloropyridin-4-yl)ureido)acetic acid (**4**, hapten *m2*) in DMSO-d_6



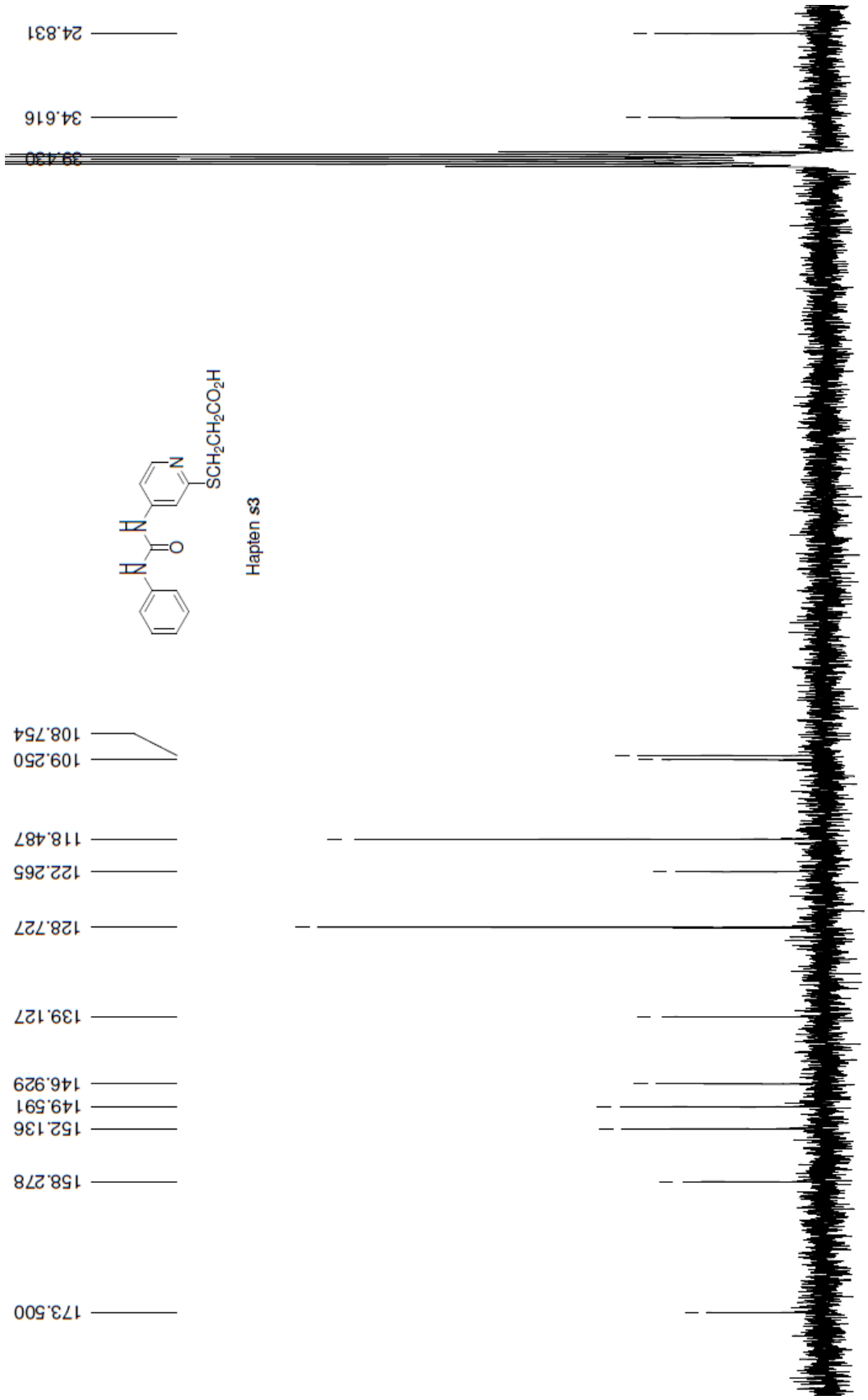
^{13}C NMR spectrum of 2-(3-(3-(2-chloropyridin-4-yl)ureido)phenyl)acetic acid (**4**, hapten *m*2) in $CDCl_3$/DMSO-d_6



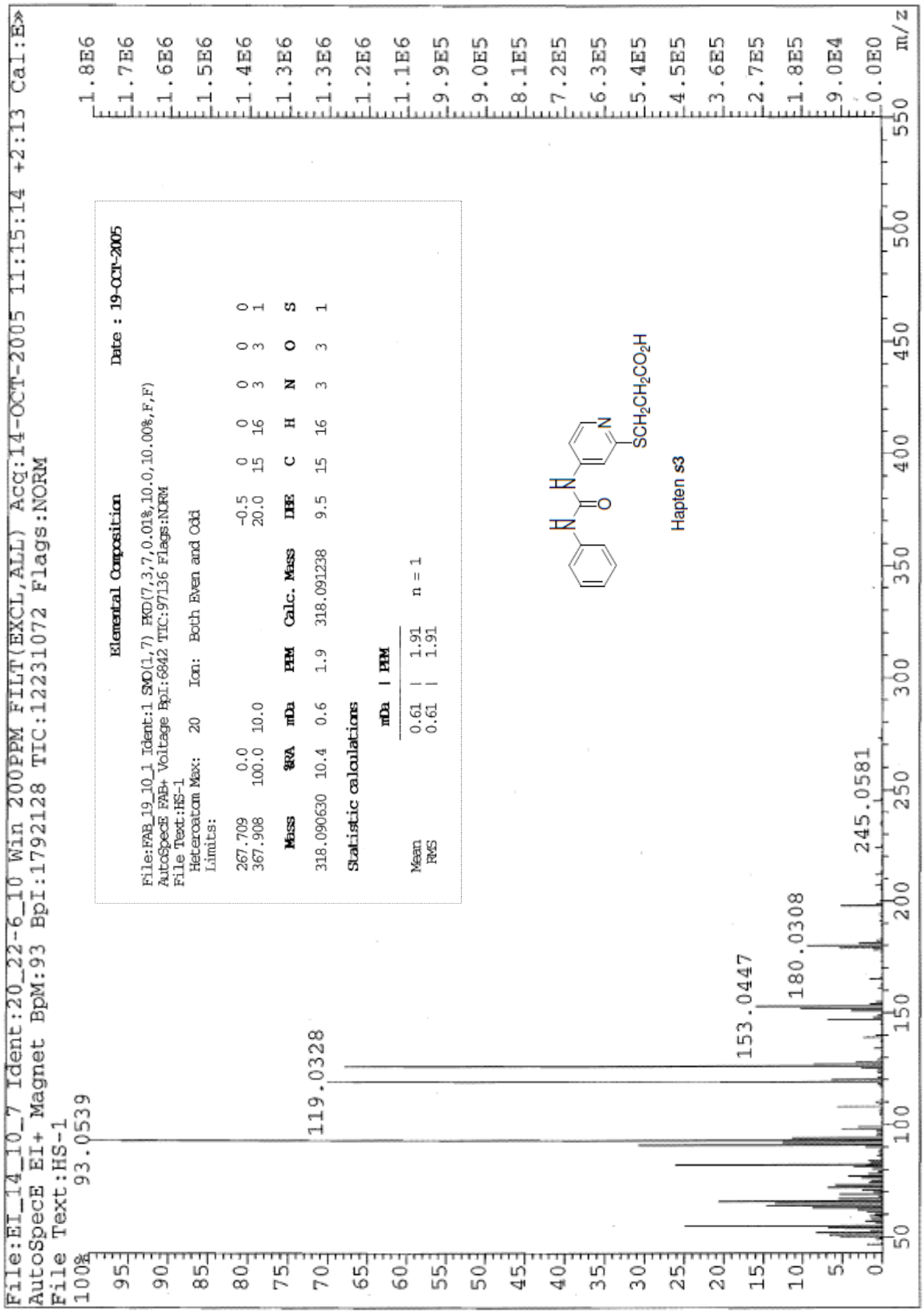
Mass spectrum of 2-(3-(3-(2-chloropyridin-4-yl)ureido)phenyl)acetic acid (**4**, hapten *m2*)



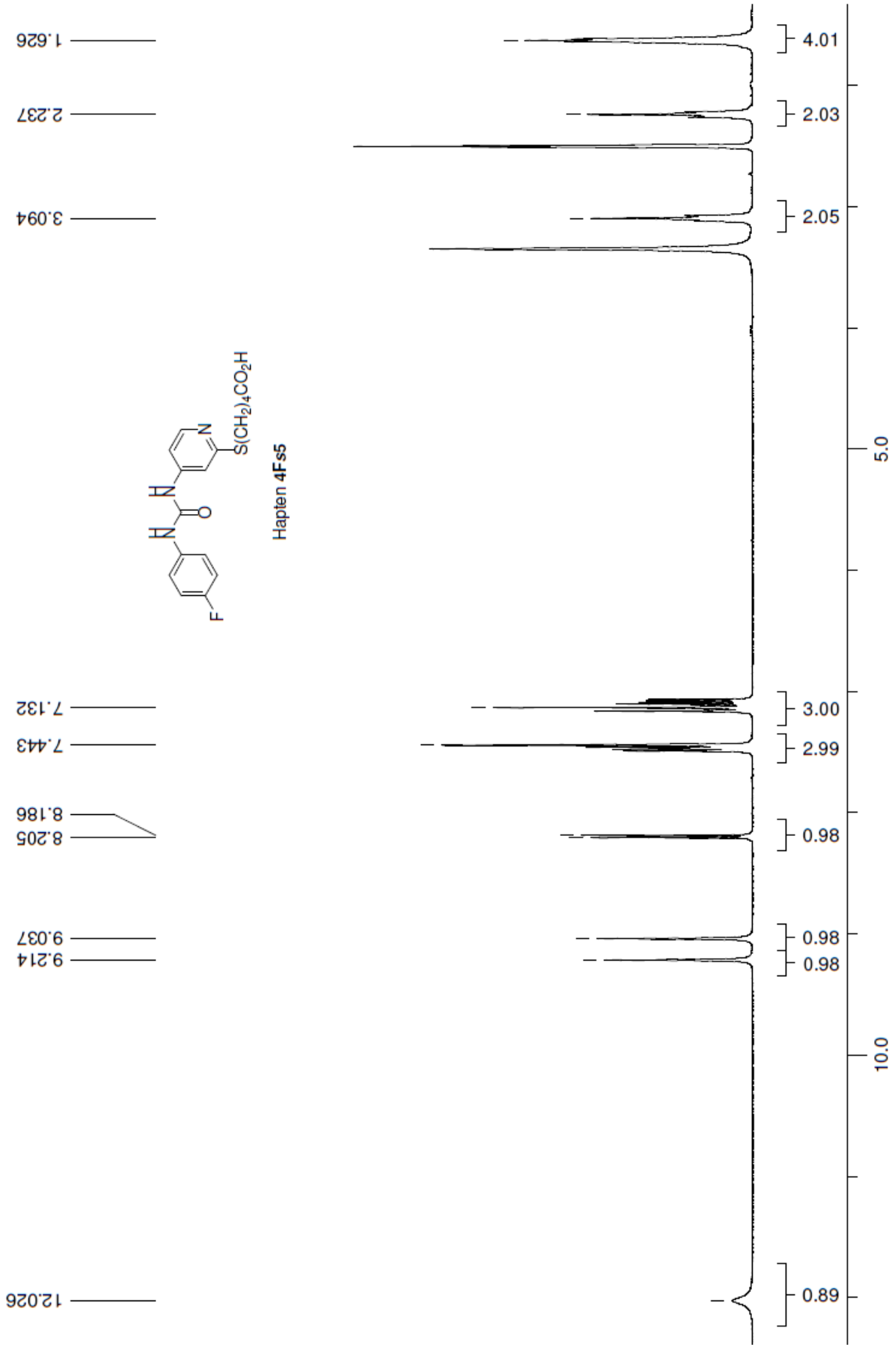
^{1}H NMR spectrum of 3-((4-(3-phenylureido)pyridin-2-yl)thio)propanoic acid (**11**, hapten *s*3) in DMSO-d_6



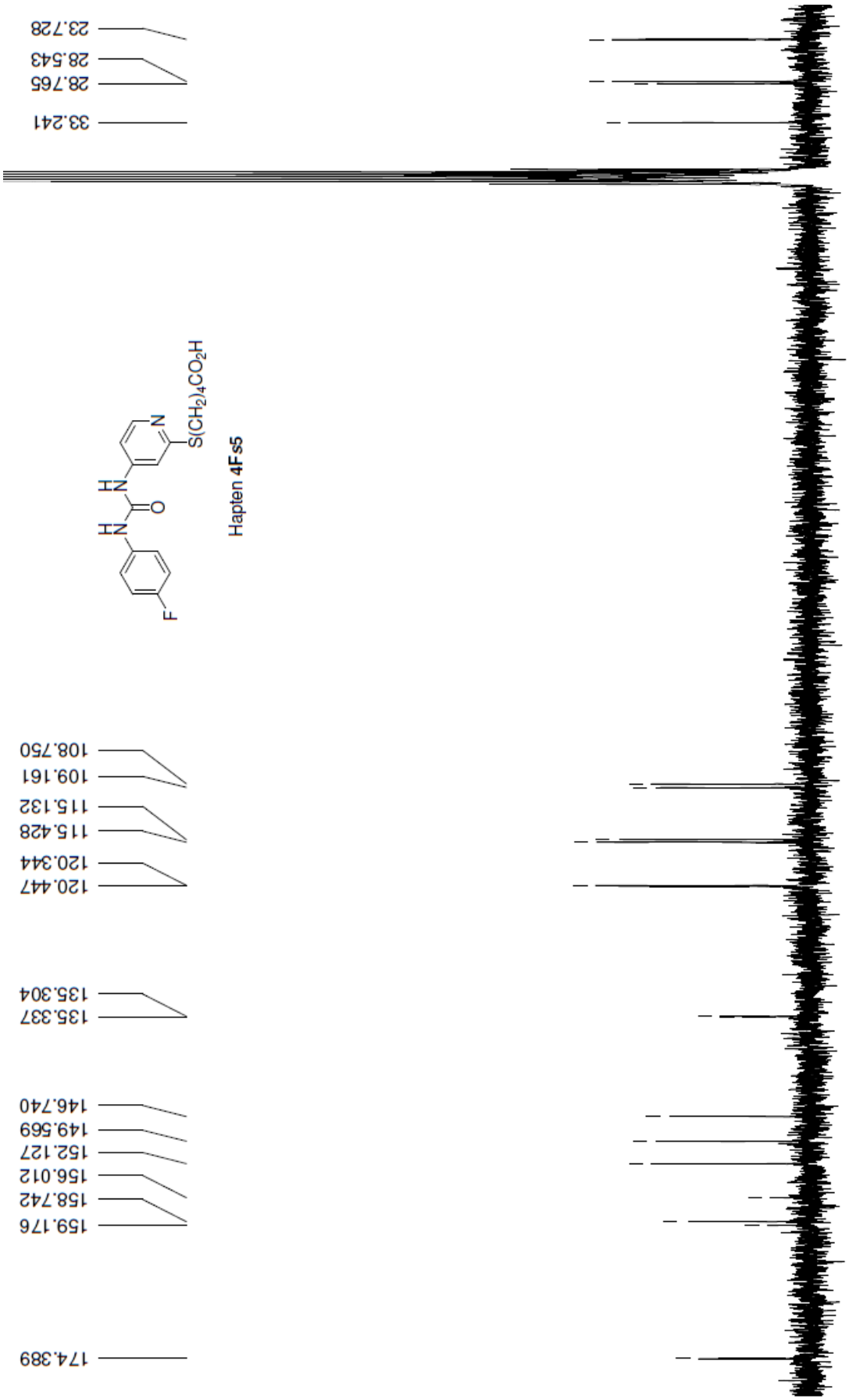
^{13}C NMR spectrum of 3-((4-(3-phenylureido)pyridin-2-yl)thio)propanoic acid (**11**, hapten *s*3) in DMSO-d_6



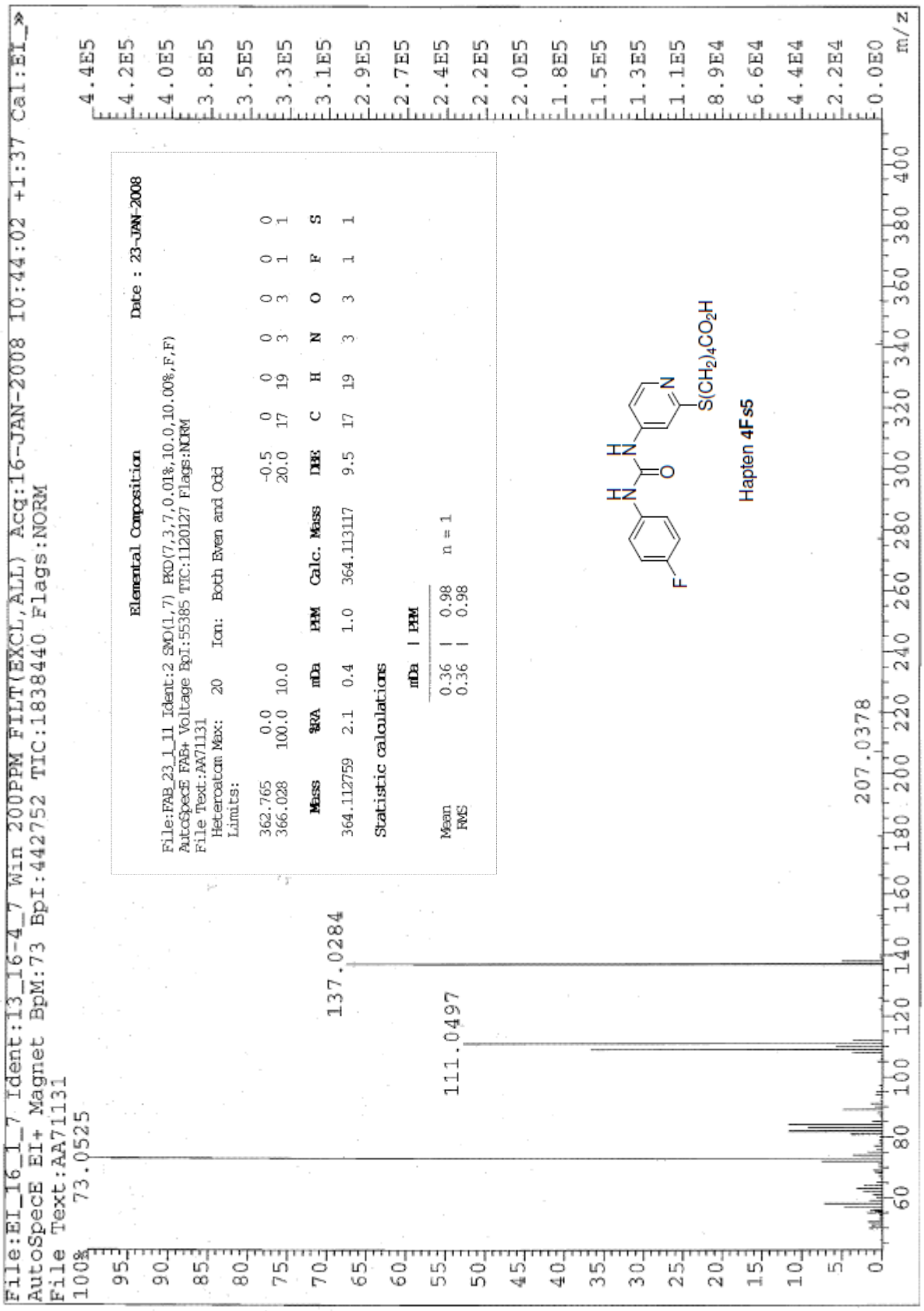
Mass spectrum of 3-((4-(3-phenylureido)pyridin-2-yl)thio)propanoic acid (**11**, hapten *s*3)



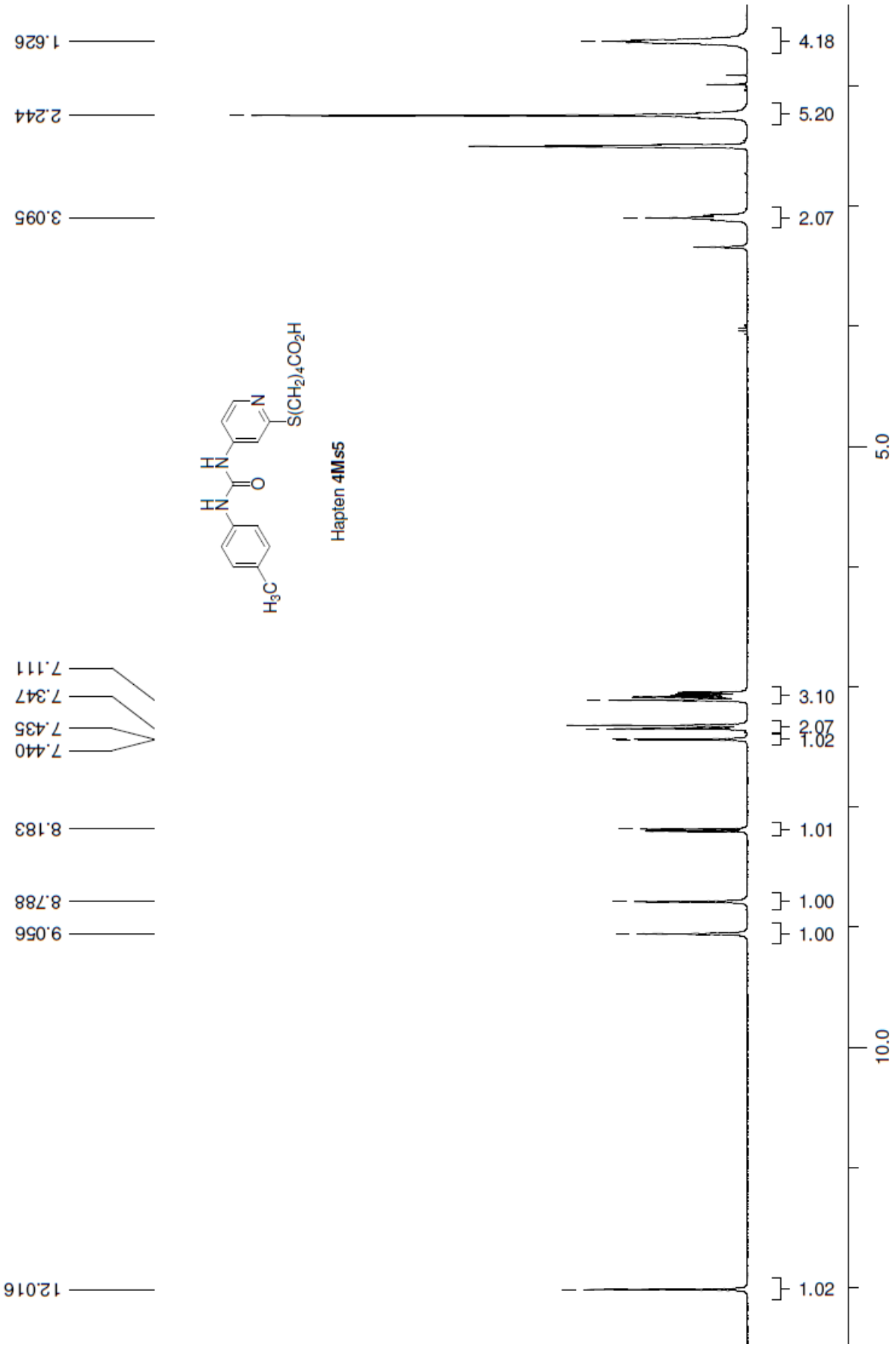
^{1}H NMR spectrum of 5-(4-(3-((4-fluorophenyl)ureido)pyridin-2-yl)thio)pentanoic acid (**15**, hapten 4F*s*5) in DMSO-d_6



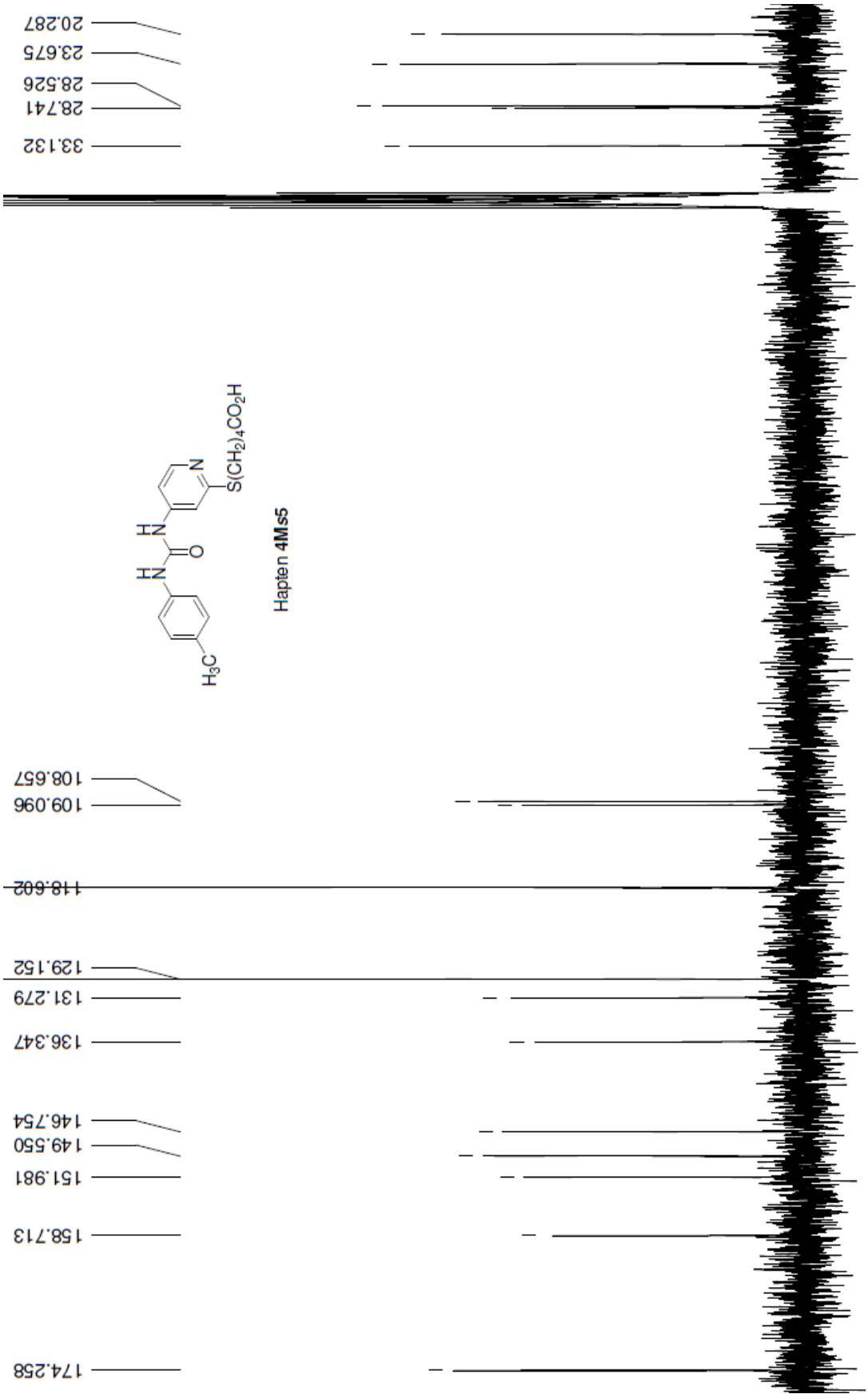
^{13}C NMR spectrum of 5-(4-(3-((4-fluorophenyl)ureido)pyridin-2-yl)thio)pentanoic acid (**15**, hapten 4Fs5) in DMSO-d_6



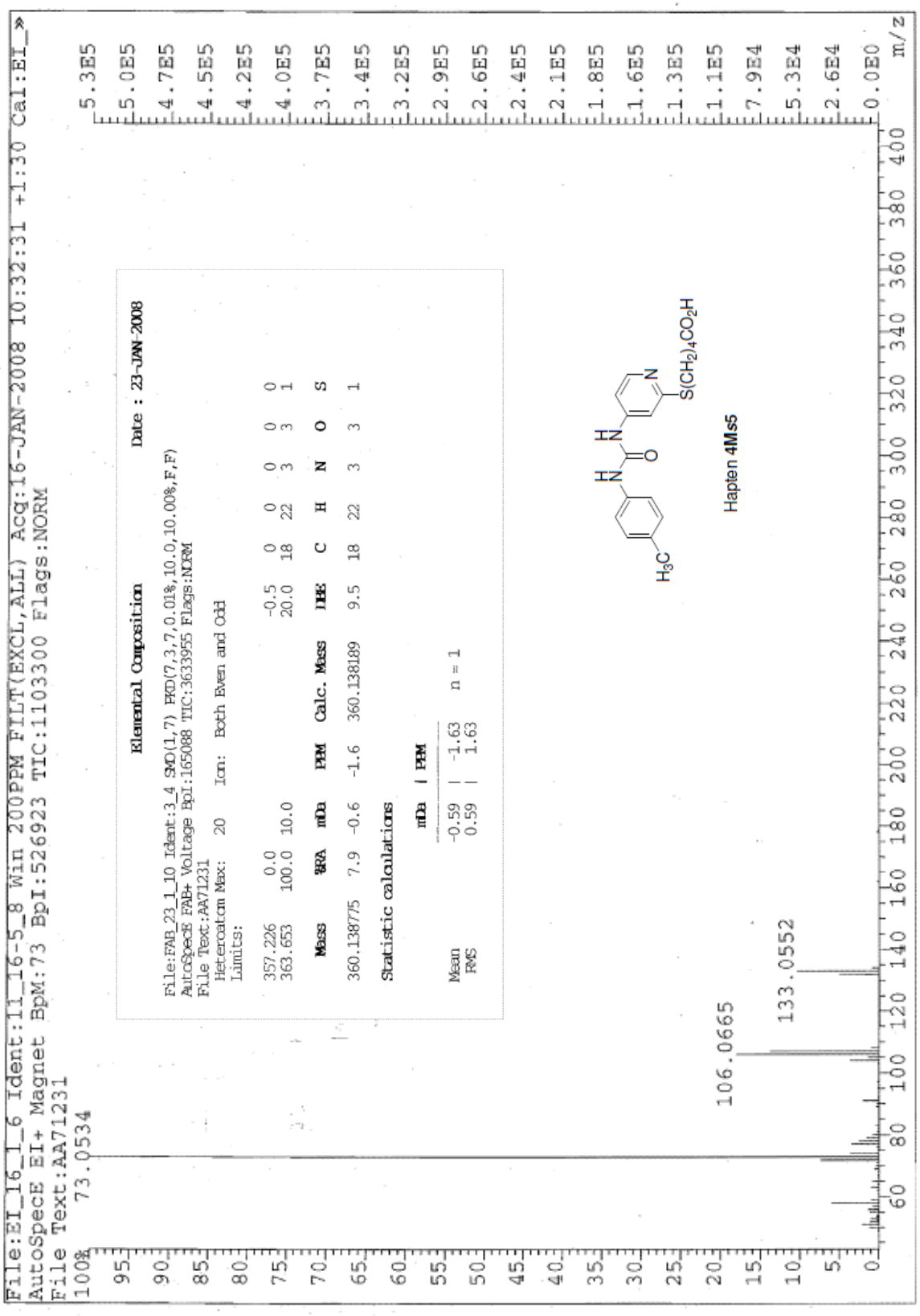
Mass spectrum of 5-(4-(3-((4-fluorophenyl)ureido)pyridin-2-yl)thio)pentanoic acid (**15**, hapten 4Fs5)



^{1}H NMR spectrum of 5-((4-(3-p-tolylureido)pyridin-2-yl)thio)pentanoic acid (**18**, hapten 4M*s*5) in DMSO-d_6



^{13}C NMR spectrum of 5-((4-(3-p-tolylureido)pyridin-2-yl)thio)pentanoic acid (**18**, hapten 4M*s*5) in DMSO-d$_6$



Mass spectrum 5-((4-(3-p-tolylureido)pyridin-2-yl)thio)pentanoic acid (**18**, hapten 4Ms5).