Electronic Supplementary Material (ESI) for Analyst.

Supplementary Information

Rationally designed haptens for highly sensitive monoclonal antibody-based immunosensing of fenhexamid

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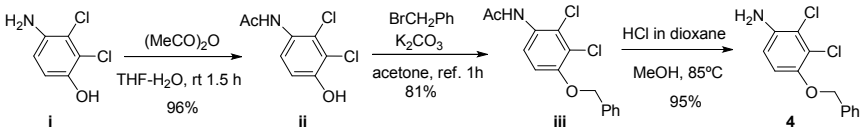
General information

Reactions involving air-sensitive compounds were conducted in oven-dried glassware under a nitrogen atmosphere. 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). 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. Melting points were determined using a Kofler hot-stage apparatus or a Büchi melting point apparatus and are uncorrected. NMR spectra were recorded 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 ^{1}H NMR spectra (7.26 ppm, 3.58 for THF-d_8 and 3.31 for CD_3OD) and to solvent carbons in ^{13}C NMR spectra (77.00 ppm and 67.57 for THF-d_8). Carbon substitution degrees were established by DEPT pulse sequences. The abbreviation used for NMR data are as follows: s =singlet, d = doublet, ddd = double double doublet, t = triplet, q = quadruplet, dt = double triplet, quint = quintuplet, br = broad, m = multiplet; Cy = cyclohexyl ring, Ph = phenyl ring, Bn = aromatic benzyl ring. Infrared (IR) spectra were measured using a Nicolet Avatar 320 FT-IR spectrometer. High resolution mass spectra (HRMS) were recorded by the electrospray (ES) ionization mode using a Q-TOF premier mass spectrometer with an electrospray source (Waters, Manchester, UK).

Preparation of 4-(benzyloxy)-2,3-dichloroaniline (4)



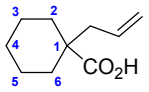
a) Acetic anhydride (589 µL, 6.24 mmol, 1.1 equiv) was dropwise added to a well stirred solution of 4-amino-2,3-dichlorophenol (1.00 g, 5.67 mmol) in THF (10 mL) and H_2O (5 mL) at room temperature. The reaction mixture was stirred at this temperature for 1.30 h and then poured into water and extracted with Et_2O. The combined organic layers were washed with an aqueous saturated solution of $NaHCO_3$ and brine and dried over anhydrous Na_2SO_4. Evaporation of the solvent at reduced pressure led to crude acetamide **ii** (1.199 mg, 96%).

b) Benzyl bromide (1.3 mL, 10.9 mmol, 2 equiv) was added to a stirred mixture of the above obtained acetamide and K_2CO_3 (3.766 g, 27.28 mmol, 5 equiv) in anhydrous acetone (37 mL) under nitrogen. The reaction mixture was stirred under reflux for 1 h, allowed to cool to room temperature, filtered through a short plug of celite, washing with acetone, and the filtrate concentrated under reduced pressure. Chromatography of the residue obtained, using hexane-EtOAc mixtures from 100:0 to

50:50, afforded *N*-(4-(benzyloxy)-2,3-dichlorophenyl)acetamide **iii** (1.37 g, 81%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.18 (1H, d, *J* = 9.3 Hz, H-5 Ph), 7.43 (6H, m, C2-6 Bn and NH), 6.92 (1H, d, *J* = 9.3 Hz, H-6 Ph), 5.16 (2H, s, CH_2), 2.23 (3H, s, CH_3).

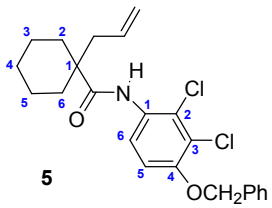
c) A solution of HCl in dioxane (4M, 12 mL, 46 mmol) was added to a solution of acetamide **iii** (942 mg, 3.036 mmol) in CH_3OH (18.9 mL) and the mixture was refluxed under anhydrous conditions for 2.30 h. The reaction mixture was cooled to room temperature, poured into H_2O and basified to pH 9-10 by the addition of 3M NaOH. The basic mixture was extracted with Et_2O and the organic extracts were washed with brine and dried with anhydrous Na_2SO_4. Evaporation of the solvent under vacuum left to the solid aniline **4** (745 mg, 95%) whose ^{1}H NMR spectrum (identical with that previously reported in the literature)[1] showed to be essentially pure and was used without further purification. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.39 (5H, m, C2-6 Bn), 6.77 and 6.61 (each 1H, AB system, *J* = 9.0 Hz, H-5 and H-6 Ph), 5.06 (2H, s, CH_2), 3.91 (2H, br s, NH_2).

Characterization data of intermediates of the synthesis of hapten FH*m*



2

1-Allylcyclohexanecarboxylic acid (**2**).[2] IR ν_{max}/cm^{-1} (NaCl) 2500-3000, 1697, 1640, 1453, 1245, 917; ^{1}H NMR (300 MHz, $CDCl_3$) δ 11.44 (1H, br s, CO_2H), 5.75 (1H, m, =CH), 5.08 and 5.03 (1H each, each m, $=CH_2$), 2.30 (2H, d, J = 7.5 Hz, CH_2CH=), 2.04 (2H, m, H-2/H-6 Cy), 1.59-120 (8H, m, 10 CH-Cy); ^{13}C NMR (75 MHz, $CDCl_3$) δ 183.5 (CO_2H), 133.3 (=CH), 117.9 ($=CH_2$), 47.1 (C1-Cy), 44.3 (CH_2-C=), 33.4 (C2/C6-Cy), 25.7 (C4-Cy), 23.0 (C3/C5-Cy).



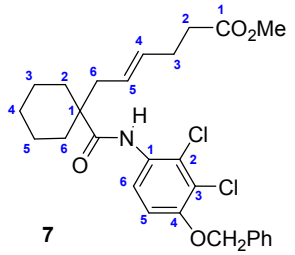
5

1-Allyl-N-(4-(benzyloxy)-2,3-dichlorophenyl)cyclohexanecarboxamide (**5**). IR ν_{max}/cm^{-1} (NaCl) 3431, 3312, 3070, 2930, 2859, 1675, 1592, 1508, 1466, 1272, 1133; ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.20 (1H, d, *J* = 9.3 Hz, H-6 Ph), 7.81 (1H, br s, NH), 7.45-7.29 (5H, m, C2-6 Bn), 6.92 (1H, d, *J* = 9.3 Hz, H-5 Ph), 5.74 (1H, m, =CH), 5.15 (2H, s, OCH_2), 5.09 and 5.04 (1H each, each m, $=CH_2$), 2.33 (2H, d, J = 7.5 Hz, CH_2CH=), 2.05

[1] a) Dinsmore, C. J.; Bergman, J. M. Inhibitors of prenyl-protein transferase. PCT Int. Appl. (2001), WO2001060815 A1, 2001-08-23. b) Practical Application of the Palladium-catalyzed Amination in Phenylpiperazine Synthesis: An Efficient Synthesis of a Metabolite of the Antipsychotic Agent Aripiprazole. *Tetrahedron,* **1998**, *54*, 4811-4818.

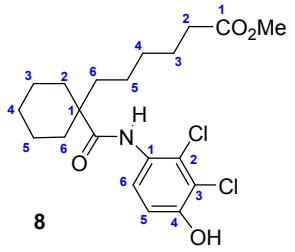
[2] Nicolai, Stefano; Piemontesi, Cyril; Waser, Jerome. A Palladium-Catalyzed Aminoalkynylation Strategy towards Bicyclic Heterocycles: Synthesis of (±)-Trachelanthamidine. *Angew. Chem. Int. Ed*., **2011,** *50*, 4680-4683.

(2H, m, H-2/H-6 Cy), 1.70-1.25 (8H, m, 8 CH-Cy); ^{13}C NMR (75 MHz, $CDCl_3$) δ 174.2 (CON), 151.4 (C4-Ph), 136.1 (C1-Bn), 133.1 (=CH), 129.5 (C1-Ph), 128.6 (C3/C5-Bn), 128.1 (C4-Bn), 127.1 (C2/C6-Bn), 123.6 (C2-Ph), 122.3 (C3-Ph), 120.1 (C6-Ph), 118.5 (=CH_2), 112.5 (C5-Ph), 71.4 (OCH_2), 47.8 (C1-Cy), 44.8 (*CH_2*-C=), 34.0 (C2/C6-Cy), 25.8 (C4-Cy), 22.8 (C3/C5-Cy); HRMS (TOF MS ES+) calcd for $C_{23}H_{26}Cl_2NO_2$ $[M+H]^+$ 418.1341, found 418.1347.



7

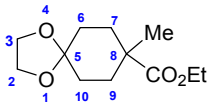
(E)-Methyl 6-(1-((4-(benzyloxy)-2,3-dichlorophenyl)carbamoyl)cyclohexyl) hex-4-enoate (**7**). IR ν_{max}/cm^{-1} (NaCl) 3432, 3310, 2929, 1737, 1679, 1594, 1510, 1470, 1272, 1026; ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.18 (1H, d, *J* = 9.3 Hz, H-6 Ph), 7.79 (1H, br s, NH), 7.47-7.32 (5H, m, C2-6 Bn), 6.92 (1H, d, *J* = 9.3 Hz, H-5 Ph), 5.37 (2H, m, H-4 and H-5), 5.09 (2H, s, OCH_2), 3.55 (3H, s, CO_2CH_3), 2.30 (4H, m, H-2 and H-3), 2.25 (2H, m, H-6), 2.05 (2H, H-2/H6-Cy), 1.70-1.25 (8H, m, 8 CH-Cy); ^{13}C NMR (75 MHz, $CDCl_3$) δ 174.3 (CON), 173.4 (CO_2), 151.4 (C4-Ph), 136.1 (C1-Bn), 132.3 (C4), 129.5 (C1-Ph), 128.6 (C3/C5-Bn), 128.1 (C4-Bn), 127.1 (C2/C6-Bn), 125.8 (C5), 125.2 (C2-Ph), 123.6 (C3-Ph), 120.1 (C6-Ph), 112.4 (C5-Ph), 71.4 (OCH_2), 51.5 (OCH_3), 48.0 (C1-Cy), 34.0 (C2/C6-Cy), 33.8 (C2), 27.8 (C3), 25.8 (C4-Cy), 22.8 (C3/C5-Cy); HRMS (TOF MS ES+) calcd for $C_{27}H_{32}Cl_2NO_4$ $[M+H]^+$ 504.1708, found 504.1710.



8

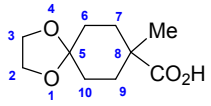
Methyl 6-(1-((2,3-dichloro-4-hydroxyphenyl)carbamoyl)cyclohexyl)hexanoate (**8**). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.95 (1H, d, *J* = 9 Hz, H-6 Ph), 7.70 (1H, br s, NH), 6.90 (1H, d *J* = 9 Hz, H-5 Ph), 6.32 (1H, br s, OH), 3.64 (3H, s, CO_2CH_3), 2.27 (2H, t, *J* = 7.2 Hz, H-2), 2.04 (2H, H-2/H-6 Cy), 1.70-1.20 (16H, m, H-3, H-4, H-5, H-6 and 8 CH-Cy); ^{13}C NMR (75 MHz, $CDCl_3$) δ 175.2 (CON), 174.3 (CO_2H), 149.4 (C4-Ph), 128.5 (C1-Ph), 123.4 (C2-Ph), 122.2 (C3-Ph), 119.1 (C6-Ph), 114.6 (C5-Ph), 51.6 (OCH_3), 47.5 (C1-Cy), 40.7 (C6), 34.4 (C2/C6-Cy), 33.9 (C2), 29.5 (C4), 25.9 (C4-Cy), 24.7 (C3), 23.6 (C5), 22.9 (C3/C5-Cy).

Characterization data of intermediates of the synthesis of haptens FH*d* and FH*h*



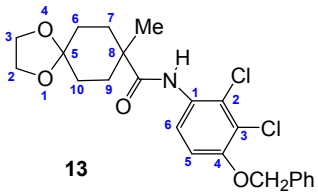
11

Ethyl 8-methyl-1,4-dioxaspiro[4.5]decane-8-carboxylate (**11**). IR ν_{max}/cm^{-1} (NaCl) 2953, 2877, 1725, 1446, 1361, 1311, 1203, 1118, 1034, 924; ^{1}H NMR (300 MHz, $CDCl_3$) δ 4.09 (2H, q, *J* = 7.1 Hz, O*CH*$_2$CH$_3$), 3.86 (4H, s, OCH$_2$CH$_2$O), 2.07 (2H, m, H-7/H-9), 1.67-1.42 (6H, m), 1.19 (3H, *t*, *J* = 7.1 Hz, OCH$_2$*CH*$_3$), 1.22 (3H, s, CH$_3$); ^{13}C NMR (75 MHz, $CDCl_3$) δ 176.8 (COO), 108.3 (C5), 64.1 (OCH$_2$CH$_2$O), 60.2 (O*CH*$_2$CH$_3$), 42.2 (C8), 32.7 (C6/C10), 31.9 (C7/C9), 25.9 (CH$_3$), 14.1 (OCH$_2$*CH*$_3$); HRMS (TOF MS ES+) calcd for $C_{12}H_{21}O_4$ $[M+H]^+$ 229.1440, found 229.1442.



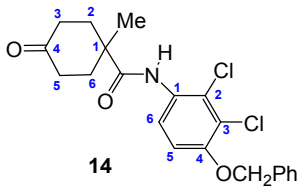
12

8-Methyl-1,4-dioxaspiro[4.5]decane-8-carboxylic acid (**12**). IR ν_{max}/cm^{-1} (KBr) 3500-2500, 2952, 2880, 1729, 1698, 1467, 1453, 1244, 1119, 1089, 1038; ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.93 (4H, s, OCH$_2$CH$_2$O), 2.13 (2H, m, H-7/H-9), 1.69-1.65 (4H, m), 1.57-1.31 (m, 2H), 1.25 (3H, s, CH$_3$); ^{13}C NMR (75 MHz, $CDCl_3$) δ 183.7 (COOH), 108.4 (C5), 64.2 (OCH$_2$CH$_2$O), 42.2 (C8), 32.6 (C6/C10), 31.9 (C7/C9), 25.8 (CH$_3$). HRMS (TOF MS ES+) calcd for $C_{10}H_{20}NaO_4$ $[M+Na]^+$ 223.0946, found 223.0947.



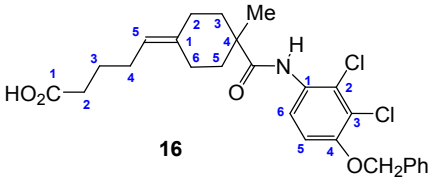
13

N-(4-(Benzyloxy)-2,3-dichlorophenyl)-8-methyl-1,4-dioxaspiro[4.5]decane-8-carboxamide (**13**). IR ν_{max}/cm^{-1} (NaCl) 3432, 3306, 2951, 2880, 1662, 1596, 1509, 1274, 1116, 1034; ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.12 (1H, d, *J* = 9 Hz, H-6 Ph), 7.85 (1H, br s, NH), 7.43-7.28 (5H, m, C2-6 Bn), 6.90 (1H, d, *J* = 9 Hz, H-5 Ph), 5.13 (2H, s, OCH$_2$), 3.93 (OCH$_2$CH$_2$O), 2.15 (2H, H-7/H-9), 1.72 (m, 6H), 1.31 (s, CH$_3$); ^{13}C NMR (75 MHz, $CDCl_3$) δ 174.9 (CON), 151.5 (C4-Ph), 136.1 (C1-Bn), 129.4 (C1 Ph), 128.6 (C3/C5-Bn), 128.1 (C4-Bn), 127.1 (C2/C6-Bn), 123.8 (C2-Ph), 122.3 (C3-Ph), 120.2 (C6-Ph), 112.4 (C5-Ph), 108.1 (C5), 71.4 (OCH$_2$), 64.2 (OCH$_2$CH$_2$O), 43.2 (C8), 33.1 (C6/C10), 31.7 (C7/C9), 21.0 (CH$_3$); HRMS (TOF MS ES+) calcd for $C_{23}H_{26}Cl_2NO_4$ $[M+H]^+$ 450.1239, found 450.1231.



14

N-(4-(Benzyloxy)-2,3-dichlorophenyl)-1-methyl-4-oxocyclohexanecarboxamide (**14**). Mp 160-161 °C (from Et_2O-hexane). IR ν_{max}/cm^{-1} (KBr) 3231, 3090, 3009, 2948, 2903, 2864, 1709, 1644, 1572, 1510, 1472, 1454, 1289, 1037; ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.12 (1H, d, *J* = 9 Hz, H-6 Ph), 7.87 (1H, br s, NH), 7.45-7.35 (5H, m, C2-6 Bn), 6.93 (1H, d, *J* = 9 Hz, H-5 Ph), 5.17 (2H, s, OCH_2), 2.55 (2H, m, H-3/H-5), 2.41 (4H, m, H'-3/H'-5 and H-2/H-6), 1.85 (2H, m, H'-2/H'-6), 1.45 (s, CH_3); ^{13}C NMR (75 MHz, $CDCl_3$) δ 210.1 (C4), 173.9 (CON), 151.9 (C4-Ph), 136.0 (C1-Bn), 128.9 (C1 Ph), 128.6 (C3/C5-Bn), 128.2 (C4-Bn), 127.1 (C2/C6-Bn), 124.2 (C2-Ph), 122.5 (C3-Ph), 120.5 (C6-Ph), 112.4 (C5-Ph), 71.4 (OCH_2), 43.2 (C1), 38.1 (C3/C5), 35.4 (C2/C6), 26.1 (CH_3); HRMS (TOF MS ES+) calcd for $C_{21}H_{22}Cl_2NO_3$ $[M+H]^+$ 406.0981, found 406.0981.



16

5-(4-((4-(Benzyloxy)-2,3-dichlorophenyl)carbamoyl)-4-methylcyclohexylidene)pentanoic acid (**16**). IR ν_{max}/cm^{-1} (NaCl) 3500-2400, 3430, 3033, 2928, 1707, 1593, 1509, 1469, 1273; ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.18 (1H, d, *J* = 9.2 Hz, H-6 Ph), 7.87 (1H, br s, NH), 7.48-7.29 (5H, m, C2-6 Bn), 6.92 (1H, d, *J* = 9.2 Hz, H-5 Ph), 5.15 (2H, s, OCH_2), 5.13 (1H, t, *J* = 7.4, H-5), 2.36 (1H, m, 1H Cy), 2.34 (2H, *t*, *J* = 7.4 Hz, H-2), 2.23-2.06 (5H, m, 5H Cy), 2.06 (2H, dt, *J* = 7.2, 7.2 Hz, H-4), 1.68 (2H, quint, *J* = 7.3 Hz, H-3), 1.51 (2H, m, 2H Cy), 1.32 (3H, s, CH_3); ^{13}C NMR (75 MHz, $CDCl_3$) δ 179.1 (COOH), 175.3 (CON), 151.5 (C4-Ph), 138.2 (C1-Cy), 136.0 (C1-Bn), 129.4 (C1-Ph), 128.6 (C3/C5-Bn), 128.1 (C4-Bn), 127.1 (C2/C6-Bn), 123.8 (C2-Ph), 122.3 (C3-Ph), 121.3 (C-5), 120.2 (C6-Ph), 112.4 (C5-Ph), 71.4 (OCH_2), 44.4 (C4-Cy), 37.0 (C3-Cy), 36.2 (C5-Cy), 33.3 (C2), 32.9 (C2-Cy), 26.4 (C-4), 26.1 (CH_3), 24.8 (C3), 24.7 (C6-Cy); HRMS (TOF MS ES+) calcd for $C_{26}H_{29}Cl_2NNaO_4$ $[M+Na]^+$ 512.1371, found 512.1375.

Hapten activation and conjugation

The hapten (1 equiv) and DSC (1.1 equiv) were dissolved in anhydrous acetonitrile (1 mL per 0.1 mmol of hapten) under nitrogen in an ice-water bath. Et_3N (3.0 equiv) was then added and the resulting mixture was stirred at 0 °C until complete consumption of the starting material (*ca*. 1–2 h, TLC using $CHCl_3$/MeOH 9:1 as eluent). The reaction mixture was diluted with $CHCl_3$, washed with brine, and dried over anhydrous Na_2SO_4. The residue that was obtained after evaporation of the solvent was purified by chromatography, eluting with $CHCl_3$/MeOH mixtures from 100:0 to 95:5, to give the corresponding NHS esters in moderate yields.

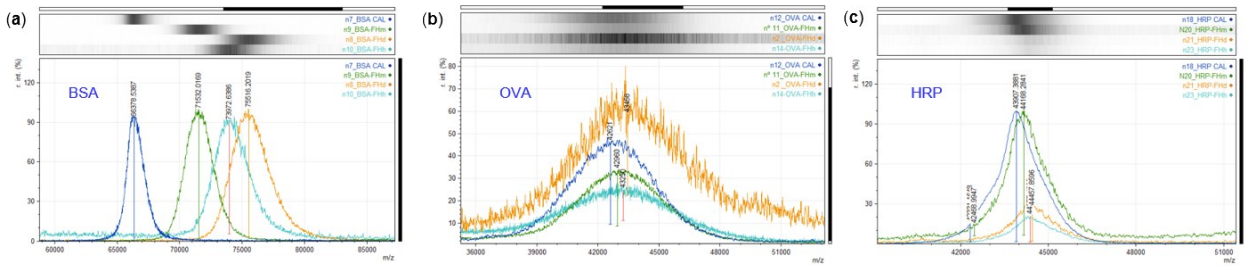
FH*m*-NHS ester: ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.10 (1H, d, *J* = 9 Hz, H-6 Ph), 7.72 (1H, br s, NH), 6.96 (1H, d, *J* = 9 Hz, H-5 Ph), 5.64 (1H, br s, OH), 2.81 (br s, 4H, $COCH_2CH_2CO$), 2.57 (2H, t, *J* = 7.2 Hz, H-2), 2.05 (2H, m, H-2/H-6 Cy), 1.75–1.15 (16H, m).

FH*d*-NHS ester: ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.09 (1H, d, *J* = 9.3 Hz, H-6 Ph), 7.76 (1H, br s, NH), 6.96 (1H, d, *J* = 9.3 Hz, H-5 Ph), 5.67 (1H, br s, OH), 5.12 (1H, t, *J* = 7.5, H-5), 2.84 (br s, 4H, $COCH_2CH_2CO$), 2.60 (2H, *t*, *J* = 7.5 Hz, H-2), 2.37 (1H, m, 1H Cy), 2.22–2.10 (7H, m, 5H Cy and H-4), 1.79 (2H, quint, *J* = 7.5 Hz, H-3), 1.54 (2H, m, 2H Cy), 1.32 (3H, s, CH_3).

FH*h*-NHS ester: A *ca.* 60:40 mixture of epimers was obtained. ^{1}H NMR (300 MHz, $CDCl_3$) (only signals of one of the epimers are given) δ 8.07 (1H, d, *J* = 9 Hz, H-6 Ph), 7.74 (1H, br s, NH), 6.93 (1H, d, *J* = 9.1 Hz, H-5 Ph), 5.91 (1H, br s, OH), 2.84 (br s, 4H, $COCH_2CH_2CO$), 2.62 (2H, *t*, *J* = 7.2 Hz, H-2), 2.22 (2H, m, H-3/H-5 Cy), 1.80–1.65 (6H, m, H-3, H-5 and 2H Cy), 1.5-1.03 (7H, m, 7H Cy), 1.31 (3H, s, CH_3).

The purified active esters of the haptens were dissolved in dry *N*,*N*-dimethylformamide and slowly added to the corresponding protein solution in 50 mM carbonate–bicarbonate buffer, pH 9.6. BSA, OVA, and HRP were used as carriers for immunogen, coating conjugate, and enzyme tracer preparation, respectively. For BSA coupling, 30 µmoles of pure active ester were applied per µmole of carrier protein, whereas for OVA conjugation 3 µmoles of activated hapten were used per mole of protein. Enzyme tracers were obtained by adding a 10-fold molar excess of pure activated hapten to the HRP solution. Conjugation was carried out at rt during 2 h with gentle stirring, and bioconjugates were purified by size exclusion chromatography using desalting columns and 100 mM phosphate buffer, pH 7.4, as eluent.

MALDI-TOF spectra of bioconjugates



	m/z	*Δm/hapten*	*Δ(m/z)*	MR
BSA	66378	-	-	-
BSA-FH*m*	71532	383.1	5154	13.4
BSA-FH*d*	75516	381.1	9138	24.0
BSA-FH*h*	73973	383.1	7595	19.8

	m/z	*Δm/hapten*	*Δ(m/z)*	MR
OVA	42621	-	-	-
OVA-FH*m*	42960	383.1	339	0.9
OVA-FH*d*	43458	381.1	837	2.2
OVA-FH*h*	43250	383.1	629	1.6

	m/z	*Δm/hapten*	*Δ(m/z)*	MR
HRP	43907	-	-	-
HRP-FH*m*	44168	383.1	261	0.7
HRP-FH*d*	44457	381.1	550	1.4
HRP-FH*h*	44377	383.1	470	1.2

Figure S1. MALDI-TOF spectra of a) BSA conjugates, b) OVA conjugates, and c) HRP conjugates of novel fenhexamid haptens. (Reference proteins are depicted in blue line, FH*m* conjugates in green line, FH*d* conjugates in orange line and FH*h* conjugates in cyan line).

Monoclonal antibody generation

Immunization. BALB/c female mice (8–10 weeks old) were immunized with the BSA conjugates by intraperitoneal injections. Doses consisted of an emulsion of 100 µL of PB containing 100 µg of protein conjugate and 100 µL of Freund's adjuvant. The first dose contained complete Freund's adjuvant, and subsequent doses were given at weeks 3 and 6 using incomplete Freund's adjuvant. After a resting period of at least 3 weeks from the last injection with adjuvant and four days before cell fusion, mice received a booster intraperitoneal injection of 200 µL of a 1:1 mixture of PBS and a solution containing 100 µg of BSA-hapten conjugate in PB.

Cell fusion and culture. P3-X63/Ag 8.653 murine myeloma cells were cultured in high-glucose DMEM supplemented with 2 mM alanylglutamine, 1 mM MEM nonessential amino acids, and 25 µg/mL gentamycin (referred to as s-DMEM) and containing 10% (v/v) foetal bovine serum (FBS). Just before spleen extraction, mouse blood was collected by heart puncture and the serum was diluted 1/10 with storage buffer and kept at 4 °C. After cytolysis of red blood cells by osmotic shock, mouse spleen lymphocytes were fused with myeloma cells at a 4:1 ratio using 1 mL of PEG 1500 as the fusing agent. The fused cells were distributed in 96-well culture plates at a density between 1.5×10^5 and 2.5×10^5 cells per well in 100 µL of s-DMEM with 15% FBS. Sixteen hours after plating, 100 µL of HAT selection medium was added to each well.

Hybridoma selection and cloning. Twelve days after fusion, hybridoma culture supernatants were screened by differential indirect cELISA with 1.0 µg/mL homologous OVA coating conjugate. RAM–HRP conjugate from Dako (Glostrup, Denmark) was employed to detect the immunological reaction. The signal of each blank assay was compared with the corresponding competitive result when 0.1 µM

fenhexamid was used as competitor. Supernatants affording a high ratio between the absorbance of both assays or with saturated signals were rescreened by checkerboard indirect cELISA. Homologous conjugate-coated plates (at 0.1 and 1.0 µg/mL) were employed and serial supernatant and fenhexamid dilutions were combined and assayed. Detected high-affinity antibody-producing hybridomas were cloned by limiting dilution in HT medium containing 20% FBS and 1% HFCS. Stable clones were expanded and cryopreserved in liquid nitrogen.

Table S1
Immunoassay conditions and standard curve parameters for fenhexamid determination

mAb	[mAb] (µg/L)	Tracer	[T][a] (µg/L)	A_{max}	IC_{50} (µg/L)
FH*m*#10	1000	HRP–FH *m*	30	1.63	0.14
	1000	HRP–FH *d*	30	1.48	0.14
	1000	HRP–FH *h*	30	1.59	0.16
	1000	HRP–FH *o*	100	---[b]	---
FH*m*#13	1000	HRP–FH *m*	10	0.86	0.05
	1000	HRP–FH *d*	10	1.12	0.07
	1000	HRP–FH *h*	10	1.00	0.09
	1000	HRP–FH *o*	100	---[c]	---
FH*m*#14	1000	HRP–FH *m*	100	1.02	0.02
	1000	HRP–FH *d*	100	0.61	0.05
	1000	**HRP–FH *h***	**30**	**1.33**	**0.02**
	1000	HRP–FH *o*	100	---	---
FH*d*#19	1000	HRP–FH *m*	10	1.42	0.39
	1000	HRP–FH *d*	3	0.82	0.60
	1000	HRP–FH *h*	10	1.08	0.52
	1000	HRP–FH *o*	100	---	---
FH*d*#132	1000	HRP–FH *m*	100	0.71	0.08
	1000	HRP–FH *d*	30	1.27	0.08
	1000	HRP–FH *h*	30	0.95	0.18
	1000	HRP–FH *o*	100	---	---
FH*d*#139	1000	HRP–FH *m*	30	1.85	0.10
	1000	HRP–FH *d*	10	1.56	0.12
	1000	HRP–FH *h*	30	1.89	0.17
	1000	HRP–FH *o*	100	---	---
FH*h*#15	1000	HRP–FH *m*	100	1.21	0.04

		d HRP–FH			
	1000	*h* HRP–FH	30	1.19	0.04
	1000	*o* HRP–FH	100	---	---
FH*h*#122	1000	*m* HRP–FH	100	---	---
	1000	*d* HRP–FH	100	0.57	0.18
	1000	*h* HRP–FH	100	0.92	0.08
	1000	*o* HRP–FH	100	---	---
FH*h*#130	1000	*m* HRP–FH	100	0.52	0.11
	1000	*d* HRP–FH	100	---	---
	1000	*h* HRP–FH	30	0.85	0.08
	1000	*o* HRP–FH	100	---	---
FHo#22	1000	*m* HRP–FH	100	---	---
	1000	*d* HRP–FH	100	---	---
	1000	*h* HRP–FH	100	---	---
	1000	*o* HRP–FH	30	1.92	0.93
FHo#26	1000	*m* HRP–FH	100	---	---
	1000	*d* HRP–FH	100	---	---
	1000	*h* HRP–FH	100	---	---
	1000	*o* HRP–FH	10	1.29	0.66
FHo#27	1000	*m* HRP–FH	100	---	---
	1000	*d* HRP–FH	100	---	---
	1000	*h* HRP–FH	100	---	---
	1000	*o* HRP–FH	10	1.55	0.69

[a] Enzyme tracer concentration. [b] A_{max} value was lower than 0.5.

Table S2
Precision of the standard curve

Parameter	RSD (%)	
	Intra-day[a]	Inter-day[b]
A_{max}	4.2	5.5
Slope	6.9	9.5
IC_{50}	6.5	5.5

[a] n = 5. [b] n = 3.

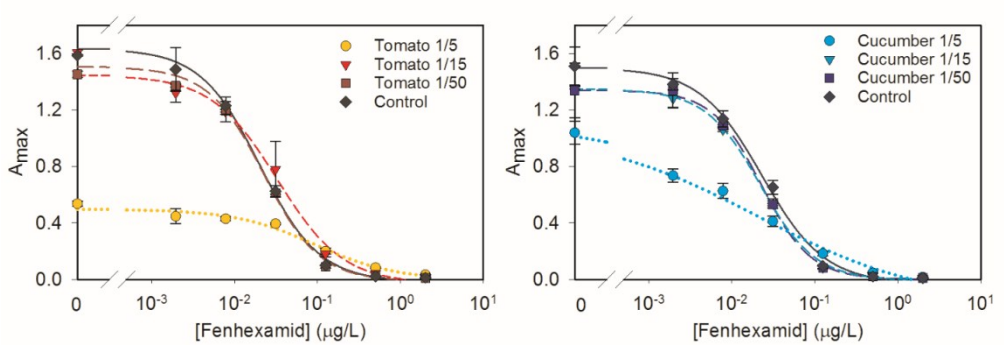


Figure S2. Matrix interferences with diluted tomato and cucumber QuEChERS extracts over the studied cELISA.

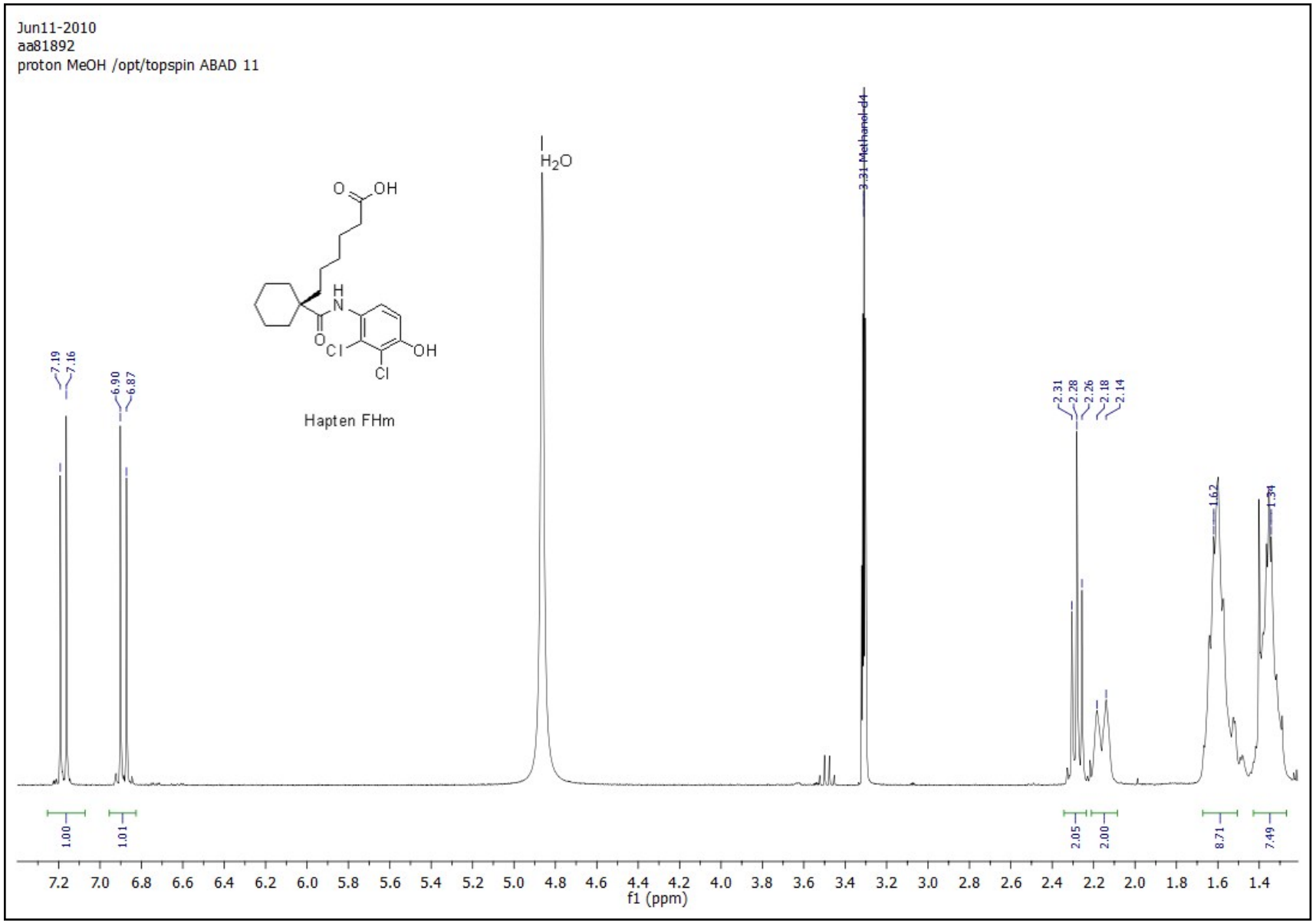
Table S3
Determination of fenhexamid residues in tomato (T) and cucumber (C) samples by the developed cELISA and a reference chromatographic method.

		[Fenhexamid] (µg/kg ± s, n=3)	
Type	Sample	cELISA	UPLC–MS/MS
In-field treated	T1	580 ± 60	650 ± 80
	T2	490 ± 70	510 ± 80
	T3	190 ± 30	180 ± 30
	T4	430 ± 70	480 ± 90
	T5	330 ± 30	310 ± 70
	C1	870 ± 30	700 ± 100
	C2	100 ± 0 300	1000 ± 100
	C3	110 ± 30	70 ± 10
	C4	150 ± 20	100 ± 10
	C5	112 ± 20	28 ± 5
Blind spiked	T6	19 ± 4	21 ± 1
	T7	70 ± 9	81 ± 2
	T8	140 ± 0 300	1300 ± 100
	T9	± 900 100	800 ± 100
	T10	± 700 100	560 ± 7
	T11	290 ± 20	290 ± 40
	T12	210 ± 30	190 ± 20
	T13	110 ± 20	120 ± 20

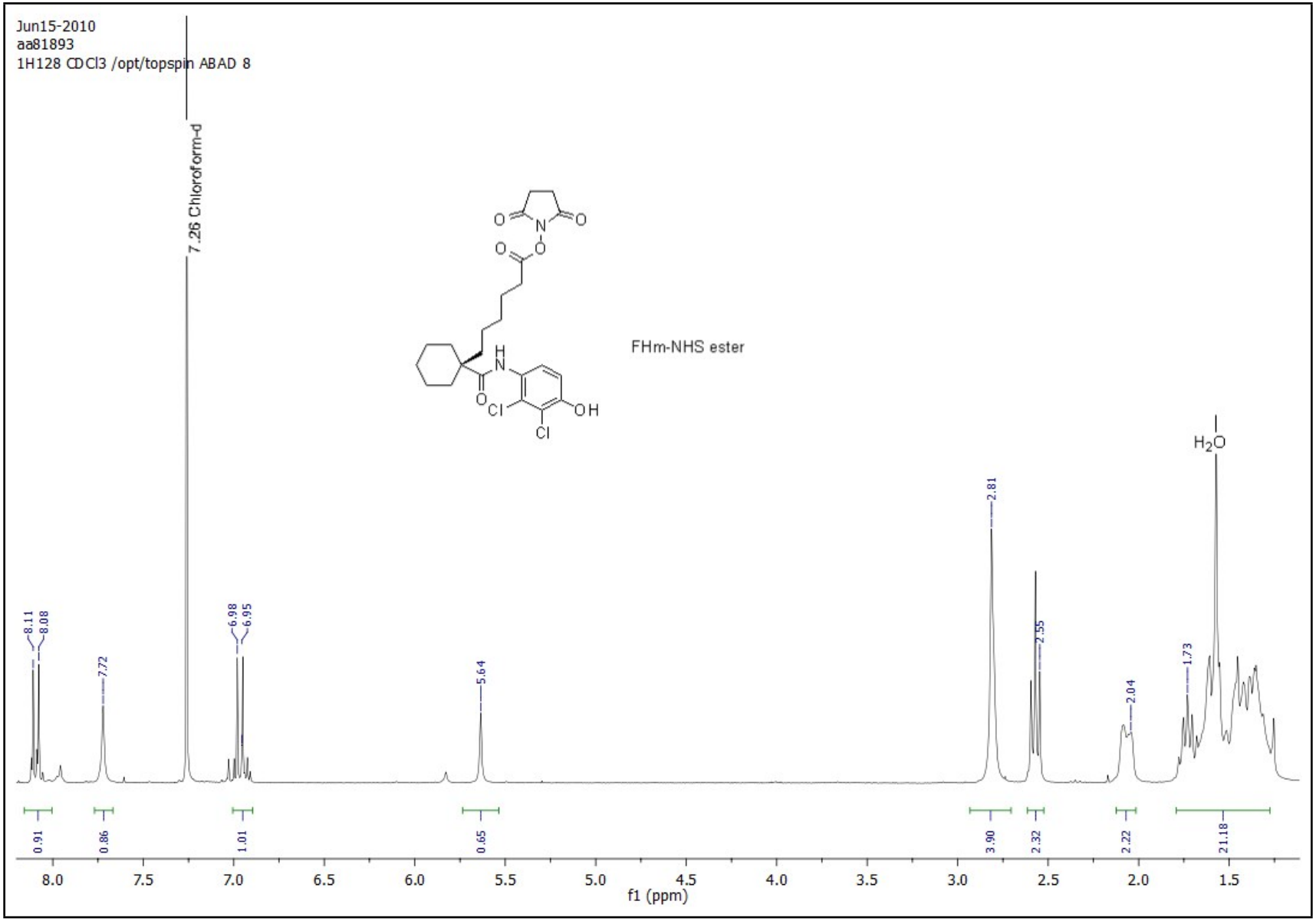
C6	100 ± 20	100 ± 3
C7	110 ± 0	1000 ± 100
C8	800 ± 100	680 ± 40
C9	90 ± 10	110 ± 20
C10	24 ± 2	24 ± 2
C11	71 ± 8	75 ± 2
C12	280 ± 30	250 ± 30
C13	130 ± 0	1100 ± 100

^{1}H NMR spectra of haptens FH*m*, FH*d* and FH*h* and their NHS-esters

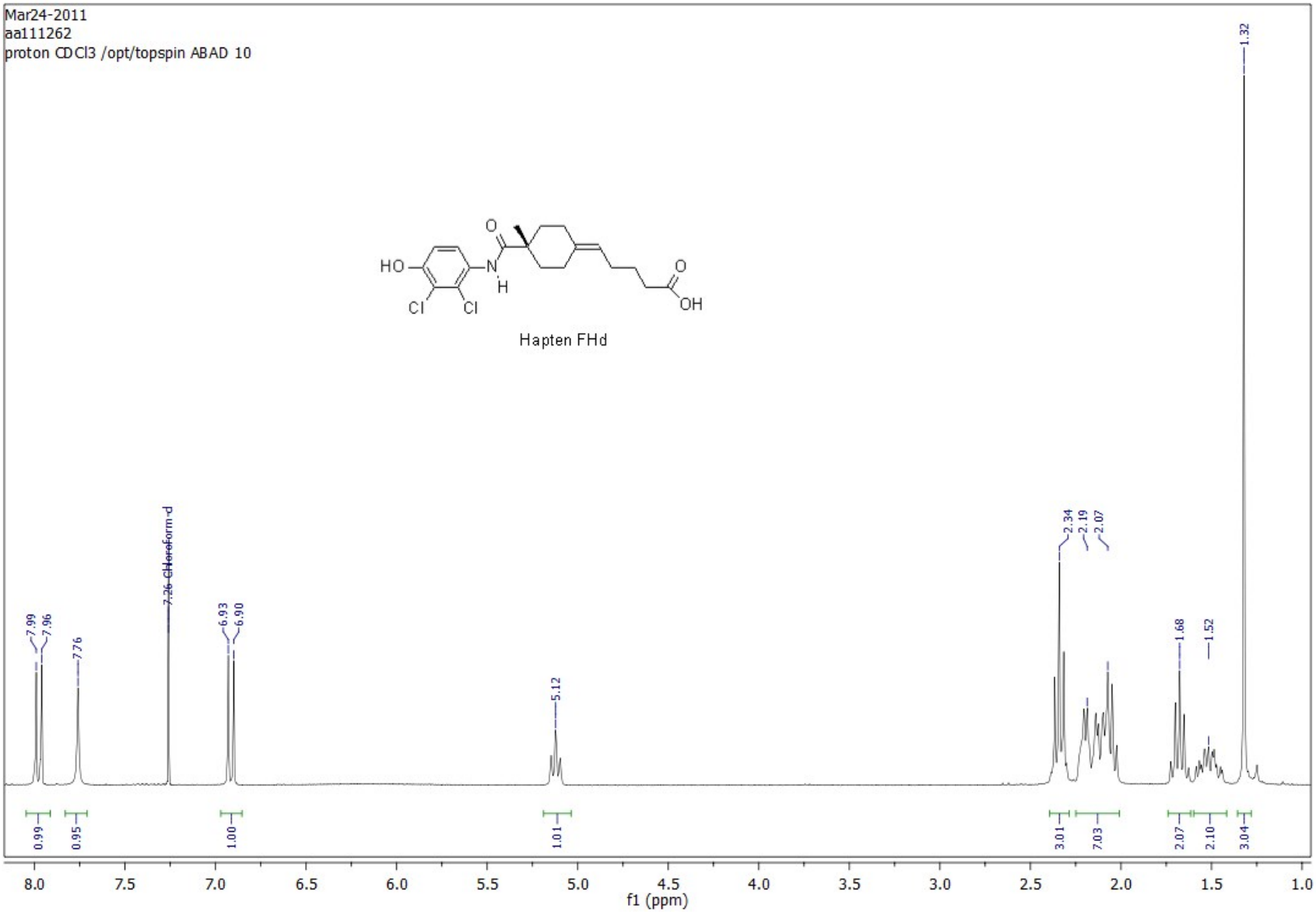
^{1}H NMR of hapten FH*m* (CD_3OD, 300 MHz)



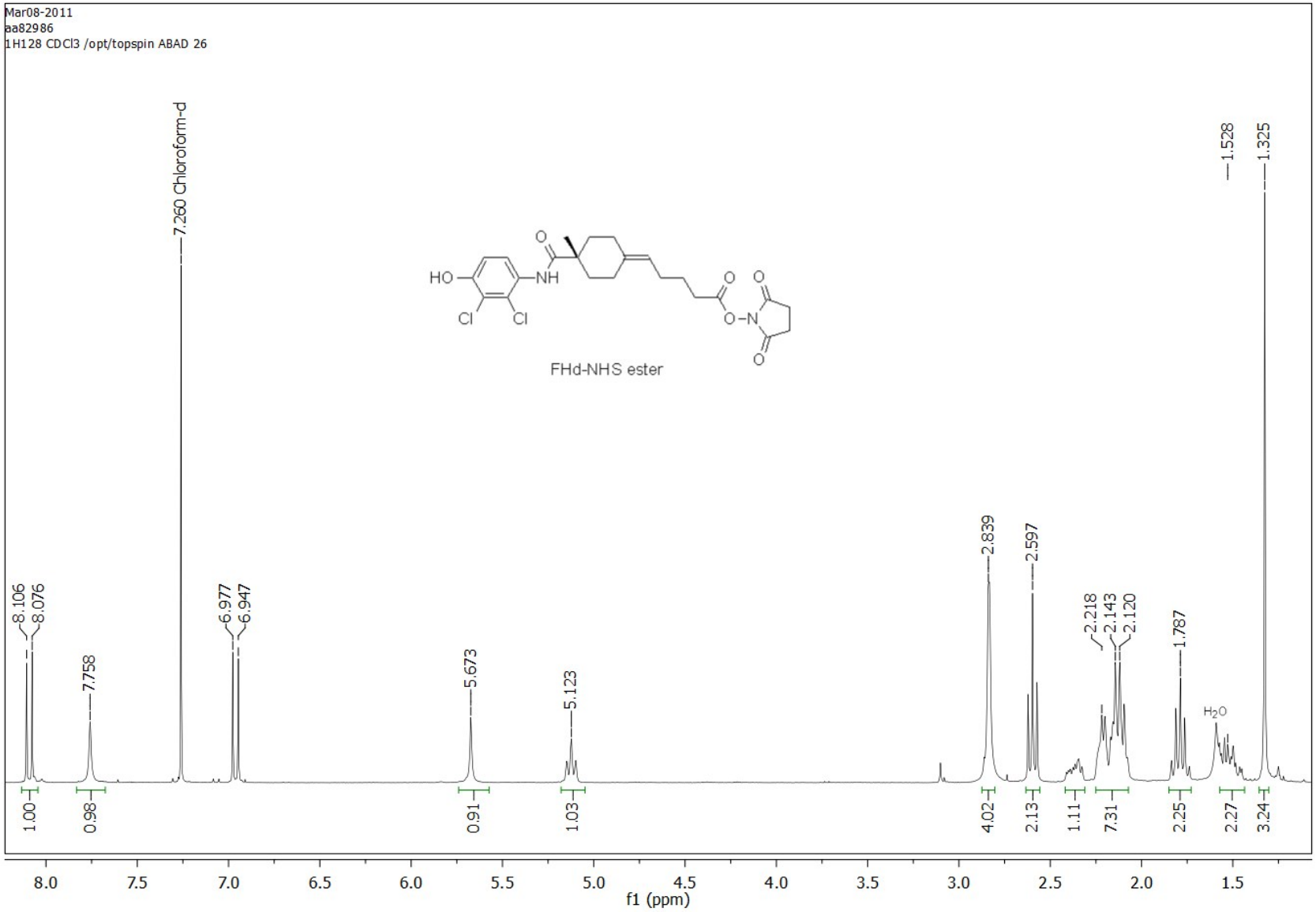
^{1}H NMR of FH*m*-NHS ester ($CDCl_3$, 300 MHz)



^{1}H NMR of hapten FH*d* ($CDCl_3$, 300 MHz)

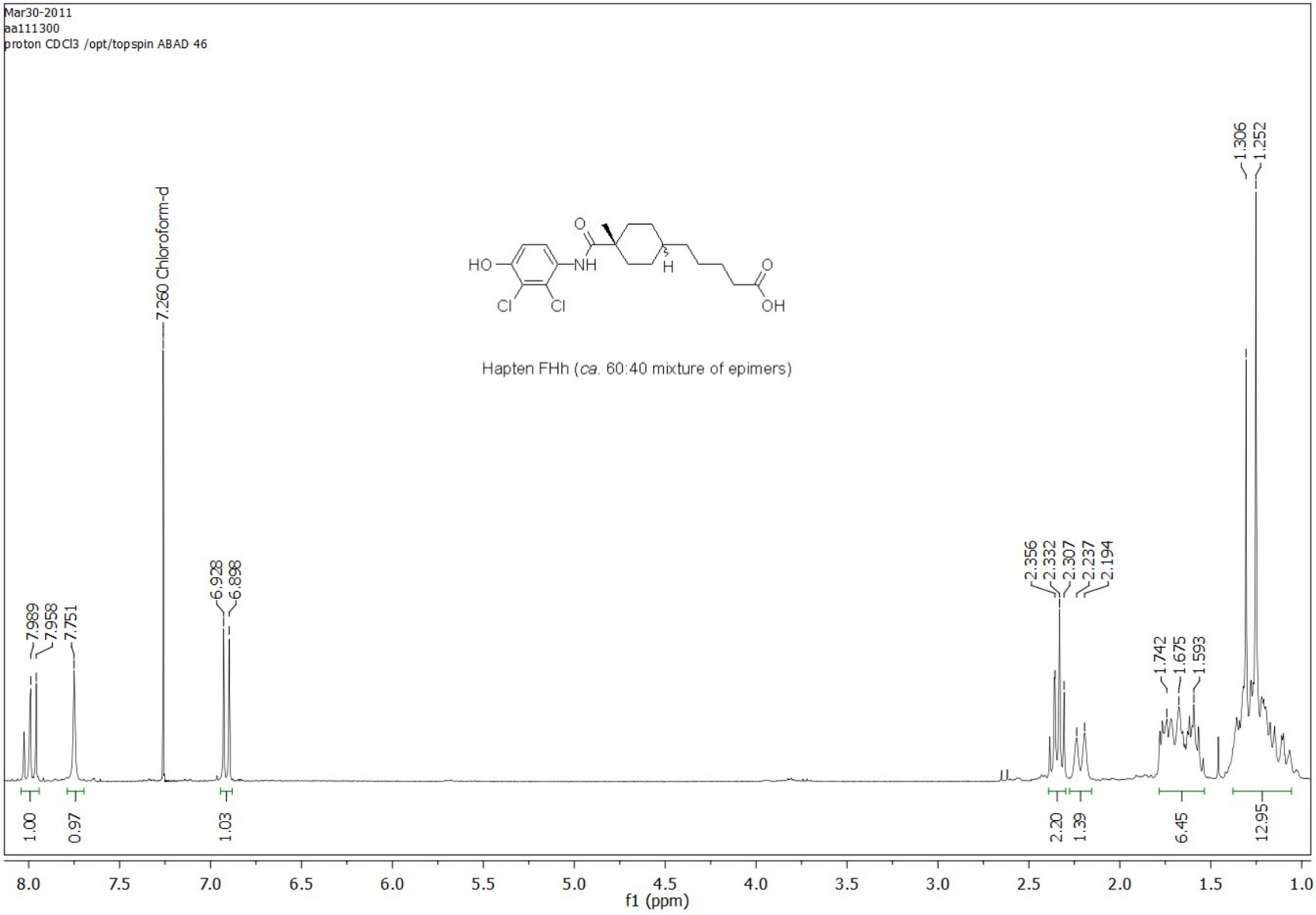


^{1}H NMR of FH*d*-NHS ester ($CDCl_3$, 300 MHz)

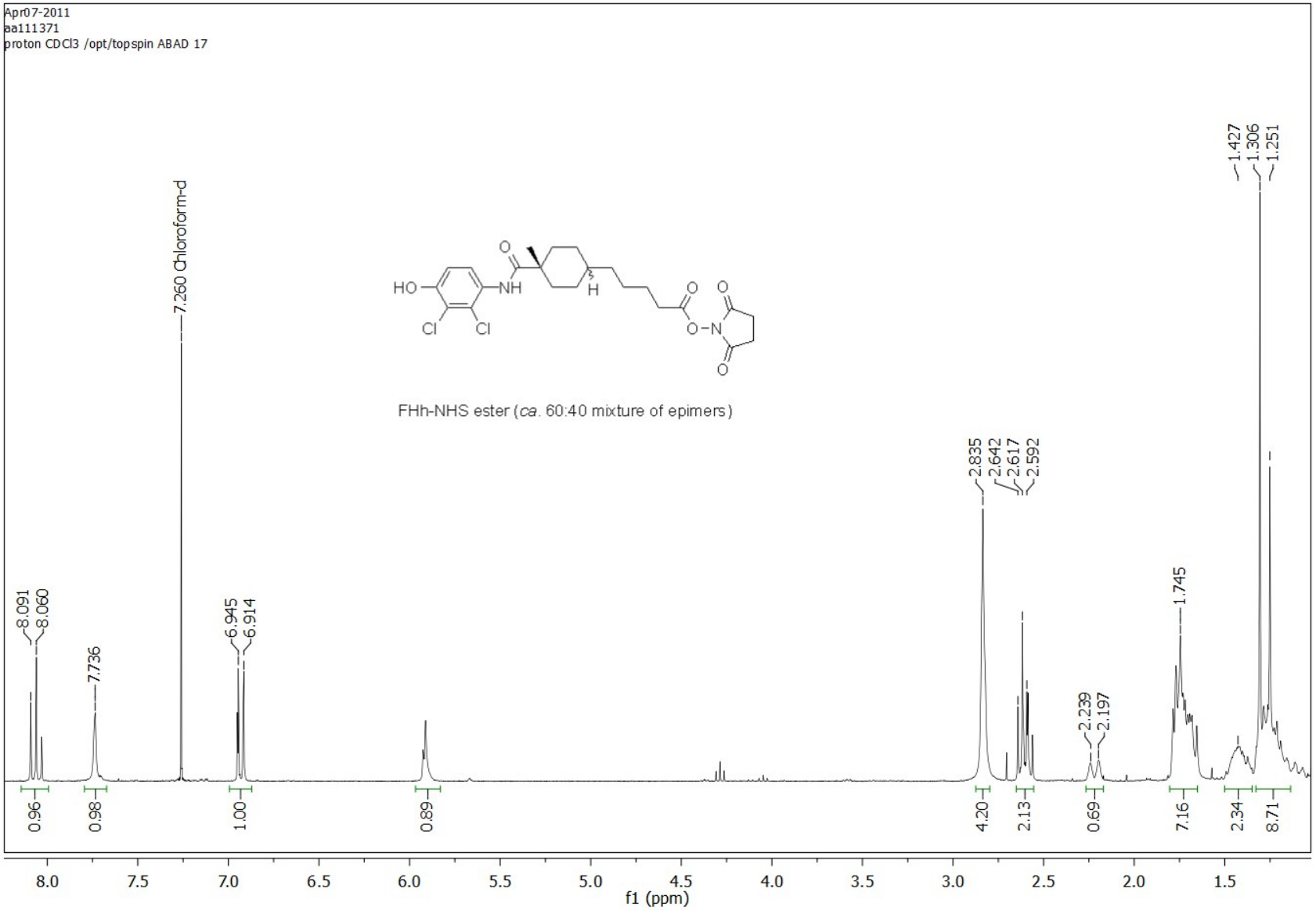


FHd-NHS ester

^{1}H NMR of hapten FH*h* ($CDCl_3$, 300 MHz)



^{1}H NMR of FH*h*-NHS ester ($CDCl_3$, 300 MHz)



FHh-NHS ester (*ca*. 60:40 mixture of epimers)