Supporting information

CLICK CHEMISTRY-ASSISTED BIOCONJUGATES FOR HAPTEN IMMUNODIAGNOSTICS

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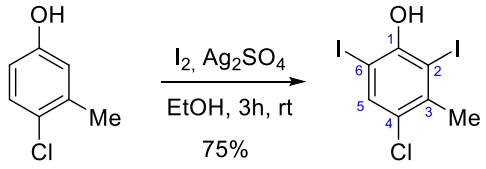
General experimental procedures and techniques

Organic solvents were dried and distilled prior use using standard techniques.[1] Et_2O and THF were distilled over Na and benzophenone under N_2 atmosphere just before being used. CH_2Cl_2 and CH_3CN were distilled from CaH_2 in the same way. MeOH was dried and stored on activated molecular sieves (3Å). DMF was distilled from CaH_2 at 7 mmHg and stored at –20 °C on 4Å molecular sieve. The remaining solvents and commercial reagents were used without prior purification. The operations with air and/or moisture-sensitive reagents were carried out under an inert atmosphere of dry N_2 or Ar, using syringes and/or cannulas, oven-dried (130 °C) glass material and freshly distilled and dried solvents. Deuterated solvents for NMR experiments were purchased from Sigma-Aldrich (Madrid) and used without further purification. Reactions under H_2 or CO pressure were performed in a TinyClave reactor from Büchi Labortechnik (Flawil, Switzerland). Microwave assisted reactions were carried out in a Discover SP reactor from CEM (Matthews, NC, EE.UU.). Reactions were monitored by thin-layer chromatography (TLC) on precoated silica plates (0.25 mm layer thickness, Silica Gel 60 F_{254}) using UV light as the visualizing agent and ethanolic phosphomolybdic acid or aqueous ceric ammonium molybdate solutions and heat as developing agents. The synthesized compounds, excluding those showing a purity above 95% by NMR, were purified by flash column chromatography using silica gel 60 (particle size 0.043–0.063 mm). Melting points were determined on a Büchi M-560 apparatus and are uncorrected. Proton and carbon nuclear magnetic resonance (^{1}H and ^{13}C NMR) spectra were recorded at rt on a Bruker Avance DPX300 spectrometer operating at 300.1 and 75.5 MHz, respectively, or on a Bruker Avance DRX500 spectrometer operating at 500.1 and 125.8 MHz, respectively. Chemical shifts (δ) are expressed in ppm recorded using the residual solvent as the internal reference in all cases [7.27/77.00 ppm, 2.50/39.51 and 3.31/49.15 for the ^{1}H/^{13}C spectra in $CDCl_3$, DMSO and MeOH, respectively (ACD/NMR Processor Academic Edition spectra processing program, version 12.0)]. Carbon substitution degrees were established by DEPT pulse sequences. A combination of COSY and HSQC experiments was used in most cases for the assignment of ^{1}H and ^{13}C chemical shifts. High resolution mass spectra (HRMS) were obtained by electrospray ionization (ESI) mode in a TripleTOF™ 5600 LC/MS/MS System (ABSciex, Framingham, MA, USA) mass spectrometer equipped with an electrospray source (Waters, Manchester, United Kingdom). The obtained data are expressed as mass/charge ratio (*m/z*).

[1] W. L. F. Armarego and D. D. Perrin, D. D. *Purification of Laboratory Chemicals*. *Butterworth Heinemann Press: Oxford* **4th Ed,** (1996).

Ochratoxin A (CAS nº 303-47-9, 403.8 Da) was purchased from Sigma/Aldrich (Madrid, Spain) and ochratoxin B (CAS nº 4825-86-9, 369.4 Da) and ochratoxin C (CAS nº 4865-85-4, 431.9 Da) were from Toronto Research Chemicals (Ontario, Canada). *o*-Phenylenediamine, Tween-20 and H_2O_2 30% (v/v) were provided by Sigma/Aldrich (Madrid, Spain). Polyclonal goat anti-rabbit immunoglobulins and goat anti-mouse immunoglobulins antibodies were obtained from Rockland Inc. (Limerick, PA, USA) and Jackson ImmunoResearch Laboratories Inc. (West Grove, PA, EE.UU), respectively. As secondary antibody, HRP–labelled polyclonal rabbit anti-mouse immunoglobulins antibody were used (Dako, Glostrup, Denmark). Costar flat-bottom high-binding 96-well polystyrene ELISA plates were from Corning (NY, USA). ELISA absorbances were read with a PowerWave HT from BioTek Instruments (Winooski, VT, EE.UU.). Microplate wells were washed with an ELx405 microplate washer also from BioTek Instruments. Nitrocellulose membranes (pore size: 15 µm) were purchased from MDI Membrane Technologies (Ambala, India) and backing cards were purchased from Kenosha (Amstelveen, The Netherlands). Cellulose sample pad and adsorbent pad were obtained from Merck-Millipore (Billerica, MA, EE.UU.) and Ahlstrom-Munksjö (Manchester, United Kingdom), respectively. 40-nm GAM–modified gold nanoparticles (OD = 10) were purchased from BBI solutions (Crumlin, United Kingdom). A ZX1010 system from Biodot (West Sussex, United Kingdom) was used for dispensing immunoreagents onto the nitrocellulose membrane. Immunostrips were cut using a CM5000 guillotine also from Biodot and manually assembled. Membranes were scanned using an EPSON V39 scanner and RGB signal was processed using ImageJ (version 1.52a) free software.

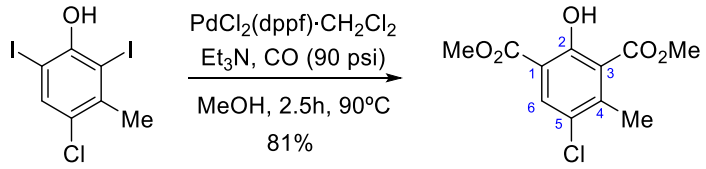
Synthesis of racemic OTα (5)[2]



4-Chloro-2,6-diiodo-3-methylphenol (**2**). Absolute EtOH (31 mL) was added to a mixture of 4-chloro-3-methylphenol (**PCMC**, **1**) (1.78 g, 12.5 mmol), iodine (6.93 g, 27.3 mmol, 2.2 equiv) and Ag_2SO_4 (7.76 g 24.9 mmol, 2 equiv) and the mixture was stirred for 3 h at rt. After the reaction was completed, the mixture was filtered to separate the salts, using $CHCl_3$ to

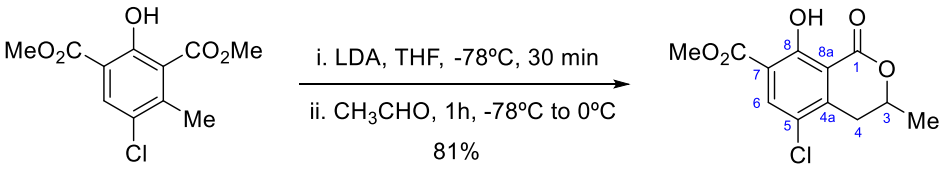
[2] D. Lopez-Puertollano, J. V. Mercader, C. Agullo, A. Abad-Somovilla and A. Abad-Fuentes. Novel haptens and monoclonal antibodies with subnanomolar affinity for a classical analytical target, ochratoxin A. *Sci. Rep.*, 2018, **8**, 9761.

wash. The filtrate was washed with a 10% aqueous solution of $Na_2S_2O_3$ and brine. After drying over anhydrous $MgSO_4$ and evaporation of the solvent in vacuo, the residue was purified by chromatography, using hexane as eluent, to obtain compound **2** (3.72 g, 75%) as a white solid. **Mp** 91.0–92.0 °C (crystallized from cold hexane). **^{1}H NMR** ($CDCl_3$, 300 MHz) δ (ppm) 2.59 (s, 3H, Me-3), 5.83 (s, 1H, OH), 7.72 (s, 1H, H-5); **^{13}C NMR** ($CDCl_3$, 75 MHz) δ (ppm) 26.8 (Me-3), 77.6 (C-6), 90.5 (C-2), 125.2 (C-4), 137.9 (C-5), 140.3 (C-3), 152.6 (C-1); **HRMS** (TOF ESI–) calcd for $C_7H_4ClI_2O$ $[M-H]^-$ 392.8046, found 392.8042.



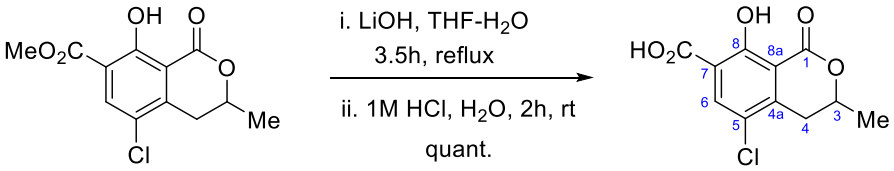
2 **3**

Dimethyl 5-chloro-2-hydroxy-4-methylbenzene-1,3-dicarboxylate (**3**). A mixture of diiodo phenol **2** (300 mg, 0.76 mmol) and $PdCl_2(dppf)\cdot CH_2Cl_2$ (64.0 mg, 0.08 mmol, 0.1 equiv) in anhydrous MeOH (9 mL) contained in a Büchi 'Tiny Clave' reactor equipped with a magnetic stirring bar, was exhaustively degassed by vacuum-filling argon cycles and cooled to 0 °C. Then, Et_3N (0.53 mL, 3.8 mmol, 5 equiv) was quickly added under a stream of argon and the mixture re-subjected to pump/purge cycles at 0 °C, first with argon and then with CO. The CO pressure was set to 90 psi and the reaction mixture was stirred at 90 °C for 2.5 h. After this time the reactor was cooled and vented, the reaction mixture was transferred to a round-bottom flask with the aid of CH_2Cl_2 and concentrated to dryness under reduced pressure. The obtained residue was suspended in Et_2O and filtered, the filtrate was washed with a 1 M aqueous solution of HCl and brine and dried over anhydrous $MgSO_4$. The residue left after evaporation of the solvent was purified by chromatography on silica gel, using hexane-EtOAc mixtures (95:5 and 90:10) as eluent, to give compound **3** (158.4 mg, 81%) as a white solid. **Mp** 66.1–67.1 °C (crystallized from cold hexane). **^{1}H NMR** ($CDCl_3$, 300 MHz) δ (ppm) 2.34 (s, 3H, Me-4), 3.95 (s, 6H, 2xOMe), 7.84 (s, 1H, H-6), 10.94 (s, 1H, OH); **^{13}C NMR** ($CDCl_3$, 75 MHz) δ (ppm) 18.0 (Me-4), 52.7 (2xOCH_3), 111.5 (C-1), 124.9 (C-3), 125.1 (C-5), 130.6 (C-6), 141.5 (C-4), 156.8 (C-2), 166.8 (CO_2-1), 169.1 (CO_2-3); **HRMS** (TOF ESI+) calcd for $C_{11}H_{12}ClO_5$ $[M+H]^+$ 259.0368, found 259.0356.



3 **4**

Methyl 5-chloro-8-hydroxy-3-methyl-1-oxoisochromane-7-carboxylate (**4**). A solution of dimethyl dicarboxylate **3** (135.6 mg, 0.52 mmol) in anhydrous THF (260 µL) was added dropwise to a solution of LDA in THF [generated from diisopropylamine (193 µL, 1.36 mmol, 2.6 equiv), BuLi (822 µL of a 1.6 M solution in hexane, 1.31 mmol, 2.5 equiv) and anhydrous THF (1.5 mL)] at –78 °C under nitrogen. The orange mixture was stirred for 20-30 min at –78 °C and then dry acetaldehyde (400 µL, 315 mg, 7.15 mmol, 14 equiv) was added. The reaction mixture was stirred at the same temperature for 10 min and at 0 °C for 1 h. After this time, the resulting yellowish reaction mixture was quenched by the addition of a 1:2 solution of AcOH in Et_2O (1 mL), then diluted with EtO_2 and washed with water, a 5% aqueous solution of $NaHCO_3$ and brine, dried over anhydrous $MgSO_4$ and concentrated in vacuo. The obtained residue (192 mg) was purified by column chromatography, using mixtures of hexane-EtOAc (100:0 and 85:15) as eluent, to give compound **4** (115.0 mg, 81%) as a white semi-solid. **^{1}H NMR** ($CDCl_3$, 300 MHz) δ (ppm) 1.58 (d, *J* = 6.4 Hz, 3H, Me-3), 2.84 (dd, *J* = 17.4, 11.6 Hz, 1H, H-4), 3.27 (dd, *J* = 17.3, 3.2 Hz, 1H, H'-4), 3.94 (s, 3H, CO_2CH_3), 4.42-4.92 (m, 1H, H-3), 8.10 (s, 1H, H-6), 12.19 (s, 1H, OH); **^{13}C NMR** ($CDCl_3$, 75 MHz) δ (ppm) 20.6 (Me-3), 32.6 (C-4), 52.6 (OCH_3), 75.1 (C-3), 111.2 (C-8a), 118.4 (C-7), 121.7 (C-5), 138.0 (C-6), 142.2 (C-4a), 161.1 (C-8), 165.0 (C-1), 167.9 (CO_2-7); **HRMS** (TOF ESI+) calcd for $C_{12}H_{12}ClO_5$ $[M+H]^+$ 271.0368, found 271.0371.

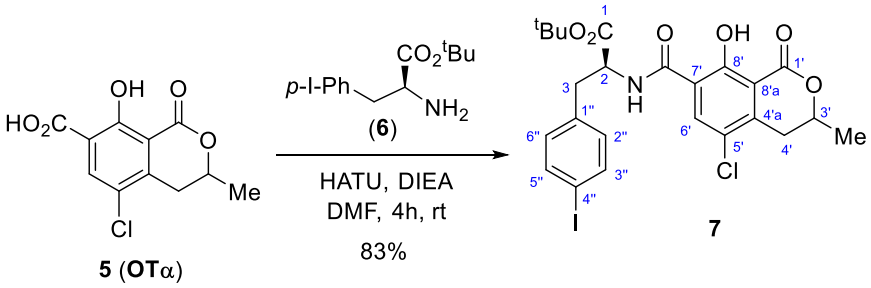


4 **5** (racemic OTα)

5-Chloro-8-hydroxy-3-methyl-1-oxoisochromane-7-carboxylic acid (**5**, racemic OTα). A solution of $LiOH{\cdot}H_2O$ (115.0 mg, 2.73 mmol, 10 equiv) in water (1.20 mL) was added to a suspension of dihydroisocoumarin **4** (74.0 mg, 0.27 mmol) in anhydrous THF (910 µL). The mixture was refluxed for 3.5 h and then cooled to 0 °C and acidified with a 1M aqueous solution of HCl (4.91 mL, 4.91 mmol, 18 equiv). The reaction mixture was stirred at rt for 2 h, diluted with water and extracted with EtOAc. The combined organic phases were washed with brine, dried over anhydrous $MgSO_4$ and concentrated under reduced pressure to afford the

acid **5** (69.9 mg, nearly quantitative yield) as a light brown amorphous solid. **^{1}H NMR** (DMSO-d_6, 300 MHz) δ (ppm) 1.44 (d, *J* = 6.2 Hz, 3H, Me-3), 2.88 (dd, *J* = 17.3, 11.6 Hz, 1H, H-4), 3.20 (dd, *J* = 17.3, 3.2 Hz, 1H, H'-4), 4.75 (m, 1H, H-3), 7.99 (s, 1H, H-6); **^{13}C NMR** (DMSO-d_6, 75 MHz) δ (ppm) 20.1 (Me-3), 32.2 (C-4), 74.4 (C-3), 112.5 (C-8a), 117.8 (C-7), 120.6 (C-5), 136.0 (C-6), 143.4 (C-4a), 160.5 (C-8), 165.4 (C-1), 167.3 (CO_2H); **HRMS** (TOF ESI+) calcd for $C_{11}H_{10}ClO_5$ $[M+H]^{+}$ 257.0211, found 257.0212.

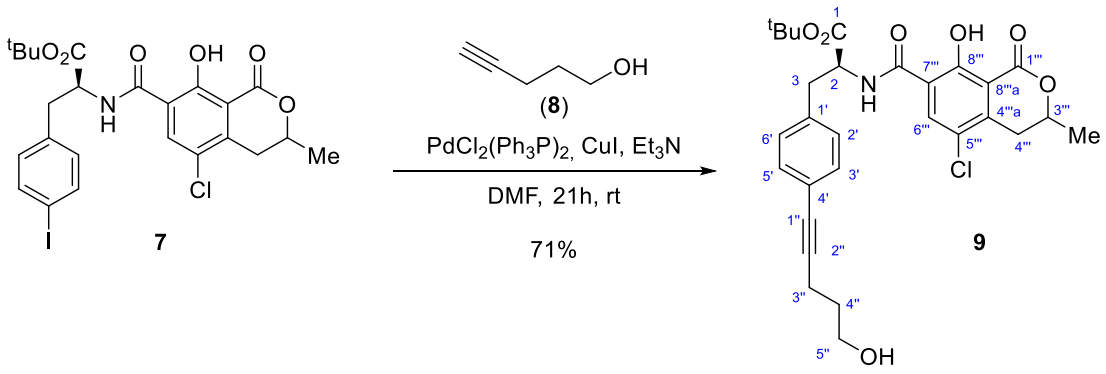
Synthesis of hapten a



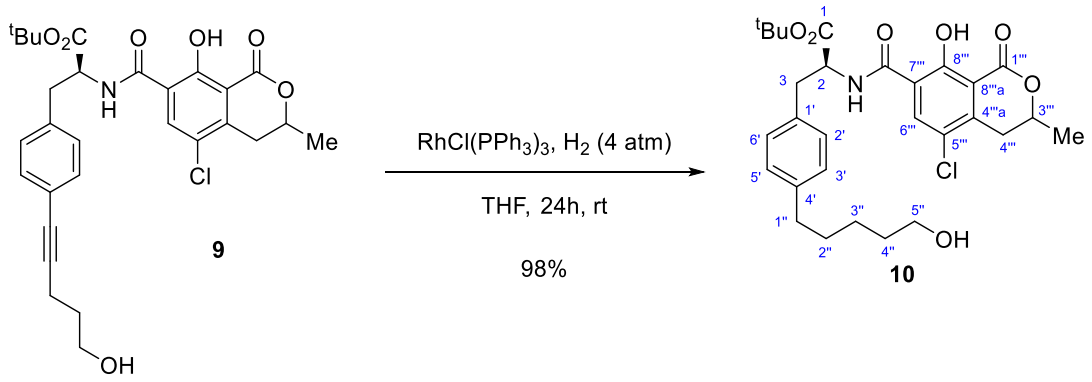
***tert*-Butyl (2*S*)-2-(5-chloro-8-hydroxy-3-methyl-1-oxoisochromane-7-carboxamido)-3-(4-iodophenyl)propanoate** (**7**). A solution of HATU (125.5 mg, 0.33 mmol, 1.5 equiv) and DIEA (80 µL, 0.44 mmol, 2 equiv) in anhydrous DMF (1.5 mL) was added successively to a solution of acid **5** (56.3 mg, 0.22 mmol) in anhydrous DMF (1.5 mL). The reaction mixture was stirred for 2 h at rt and then a solution of *tert*-butyl (*S*)-2-amino-3-(4-iodophenyl)propanoate (**6**)[3] (153 mg, 0.44 mmol, 2 equiv) and DIEA (80 µL, 0.44 mmol, 2 equiv) in anhydrous DMF was added and stirred at rt for 4 h, after which the reaction was diluted with EtOAc and washed successively with aqueous solutions of HCl (1M), LiCl (1.5%), $NaHCO_3$ (5%) and brine, dried over anhydrous $MgSO_4$ and concentrated in the rotary evaporator. The obtained residue was purified by column chromatography, using hexane-EtOAc-AcOH mixtures (100:0:0.3, 90:10:0.3 and 80:20:0.3) as eluent, to obtain compound **7** (107 mg, 83%) as a yellowish oil (a 1:1 mixture of diastereoisomers). **^{1}H NMR** ($CDCl_3$, 300 MHz) δ (ppm) 1.43 (two s, each 4.5H, CMe_3 of each diastereoisomer), 1.61 (two d, *J* = 6.4 Hz, each 1.5H, Me-3' of each diastereoisomer), 2.84 and 2.89 (two dd, *J* = 17.4, 11.6 Hz, each 0.5H, H-4' of each diastereoisomer), 3.07–3.24 (m, 2H, H_{2-} 3), 3.30 (dd, *J* = 17.4, 3.5 Hz, 1H, H'-4'), 4.77 (m, 1H, H-3'), 4.94 (dt, *J* = 7.3, 6.0 Hz, 1H, H-2), 6.96 (br d, *J* = 7.9 Hz, 2H, H-2'' and H-6''), 7.56–7.62 (m, 2H, H-3'' and H-5''), 8.44 (s, 1H, H-6'), 8.57 (m, 1H, NH), 12.78 (br s, 1H, OH); **^{13}C NMR** ($CDCl_3$, 75 MHz) δ (ppm) 20.7 (Me-3'), 28.0 (CMe_3), 32.3 (C-4'), 37.6 (C-3), 54.3 (C-2), 75.9 (C-3'), 82.6 (CMe_3), 92.4 (C-4''), 110.0 (C-8'a),

[3] The synthesis of **6** was based on K. Cheng, X. Wang and H. Yin. Small-molecule inhibitors of the TLR3/dsRNA complex. *J. Am. Chem. Soc.,* 2011, **133**, 3764–3767.

120.7 (C-7'), 123.1 (C-5'), 131.5 (C-2'' and C-6''), 136.1 (C-1''), 137.4 (C-3'' and C-5''), 138.9 (C-6'), 140.7 (C-4'a), 159.0 (C-8'), 162.1 (CONH), 169.7 (C-1'), 170.1 (C-1); **HRMS** (TOF ESI+) calcd for $C_{24}H_{26}ClINO_6$ $[M+H]^{+}$ 586.0488, found 586.0459.

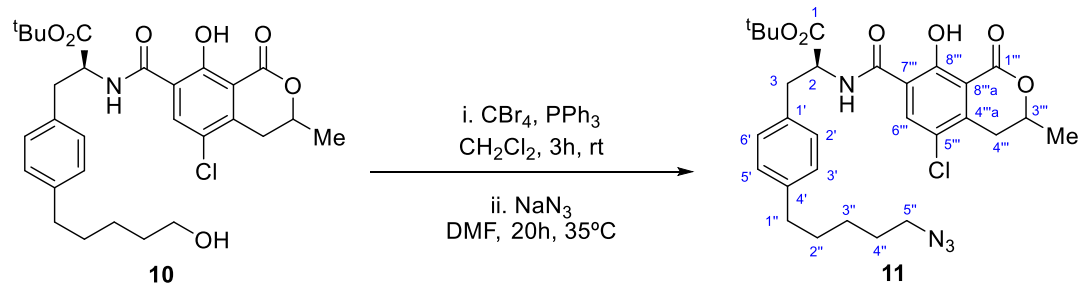


***tert*-Butyl (2*S*)-2-(5-chloro-8-hydroxy-3-methyl-1-oxoisochromane-7-carboxamido)-3-(4-(5-hydroxypent-1-yn-1-yl)phenyl)propanoate** (**9**). A mixture of iodinated derivative **7** (155.0 mg, 0.265 mmol), CuI (6 mg, 0.031 mmol, 0.12 equiv), $PdCl_2(Ph_3P)_2$ (15 mg, 0.021 mmol , 0.08 equiv) and pent-4-yn-1-ol (**8**, 67.0 mg, 0.796 mmol, 3.0 equiv) was purged by repeated vacuum-nitrogen cycles at 0 °C. Next, anhydrous DMF (2.5 mL) and Et_3N (1.3 mL, 8.58 mmol, 32 equiv) were added and the mixture was purged again. After stirring at rt for 21 h, the reaction mixture was diluted with EtOAc and washed successively with aqueous solutions of HCl (1M), LiCl (1.5%), $NaHCO_3$ (5%) and brine. The organic phase was dried over anhydrous $MgSO_4$ and concentrated under reduced pressure to give a residue, which after purification by column chromatography, using $CHCl_3$ as eluent, afforded compound **9** (101.8 mg, 71%) as a yellowish oil (a 1:1 mixture of diastereoisomers). **^{1}H NMR** ($CDCl_3$, 300 MHz) δ (ppm) 1.41 (two s, each 4.5H, CMe_3 of each diastereoisomer), 1.58 (two d, *J* = 6.4 Hz, each 1.5H, Me-3''' of each diastereoisomer), 1.83 (quin, *J* = 6.5 Hz, 2H, H_2-4''), 1.94 (br s, 1H, OH), 2.51 (t, *J* = 7.0 Hz, 2H, H_2-3''), 2.77-2.91 (m, 1H, H-4'''), 3.18 (m, 2H, H_2-3), 3.27 (dd, *J* = 17.3, 3.5 Hz, 1H, H'-4'''), 3.79 (t, *J* = 6.1 Hz, 2H, H_2-5''), 4.75 (m, 1H, H-3'''), 4.94 (dt, *J* = 7.3, 6.0 Hz, 1H, H-2), 7.12 (d, *J* = 7.9 Hz, 2H, H-3' and H-5'), 7.25-7.31 (m, 2H, H-2' and H-6'), 8.42 (s, 1H, H-6''') 8.54 (two d, *J* = 7.3, each 0.5H, NH of each diastereoisomer) 12.74 (br s, 1H, OH); **^{13}C NMR** ($CDCl_3$, 75 MHz) δ (ppm) 15.9 (C-3''), 20.6 (Me-3'''), 27.9 (CMe_3), 31.3 (C-4''), 32.2 (C-4'''), 37.9 (C-3), 54.4 (C-2), 61.7 (C-5''), 75.8 (C-3'''), 80.8 (C-1''), 82.5 (CMe_3), 89.4 (C-2''), 110.0 (C-8'''a), 120.7 (C-7'''), 122.3 (C-4'), 123.0 (C-5'''), 129.4 (C3' and C-5'), 131.4 (C2' and C-6'), 135.9 (C-1'), 138.9 (C-6'''), 140.6 (C-4'''a), 159.0 (C-8'''), 162.1 (CONH), 169.7 (C-1'''), 170.1 (C-1); **HRMS** (TOF ESI+) calcd for $C_{29}H_{33}ClNO_7$ $[M+H]^{+}$ 542.1940, found 542.1936.



9 10

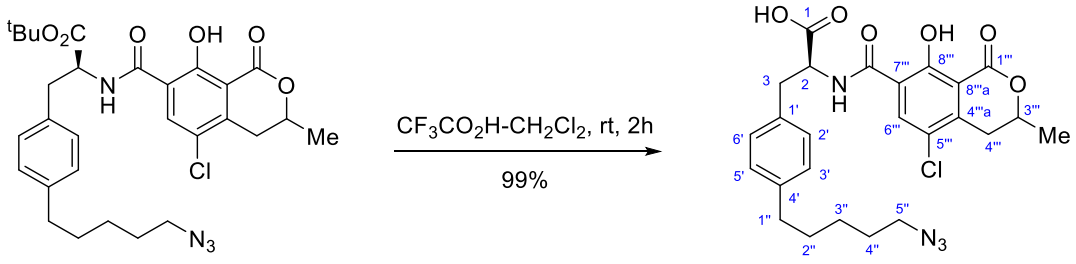
***tert*-Butyl (2*S*)-2-(5-chloro-8-hydroxy-3-methyl-1-oxoisochromane-7-carboxamido)-3-(4-(5-hydroxypentyl)phenyl)propanoate (30).** A solution of alkyne **9** (90.0 mg, 0.166 mmol) and $RhCl(PPh_3)_3$ (23.0 mg, 0.025 mmol, 0.15 equiv) in anhydrous THF (4 mL) contained in a Büchi 'Tiny Clave' reactor was purged with hydrogen. The hydrogen pressure was adjusted to 4 atm and stirred for 24 h at rt. After venting, the reaction mixture was concentrated to dryness to obtain a residue which was purified by column chromatography, using $CHCl_3$ as eluent, to obtain compound **10** (88.7 mg, 98%) as a yellowish oil (a 1:1 mixture of diastereoisomers). **^{1}H NMR** ($CDCl_3$, 300 MHz) δ (ppm) 1.36-1.40 (m, 2H, H_2-3''), 1.42 (s, 9H, CMe_3), 1.60 (m, 7H, H_2-2'', H_2-4'' and Me-3'''), 2.59 (t, *J* = 7.6 Hz, 2H, H_2-1''), 2.85 and 2.86 (two dd, *J* = 17.3, 11.6 Hz, each 0.5H, H-4''' of each diastereoisomer), 3.17 (m, 2H, H_2-3), 3.29 (dd, *J* = 17.4, 3.5 Hz, 1H, H'-4'''), 3.62 (t, *J* = 6.6 Hz, 2H, H_2-5''), 4.76 (m, 1H, H-3'''), 4.94 (dt, *J* = 7.4, 6.0 Hz, 1H, H-2), 7.05-7.14 (m, 4H, H-2', H-3', H-5' and H-6'), 8.45 (s, 1H, H-6'''), 8.53 (m, 1H, NH); **^{13}C NMR** ($CDCl_3$, 75 MHz) δ (ppm) 20.7 (Me-3'''), 25.3 (C-3''), 28.0 (*C*Me_3), 31.1 (C-4''), 32.2 (C-4'''), 32.6 (C-2''), 35.4 (C-1''), 37.7 (C-3), 54.6 (C-2), 62.9 (C-5''), 75.9 (C-3'''), 82.2 (*C*Me_3), 110.0 (C-8'''a), 120.9 (C-7'''), 123.0 (C-5'''), 128.4 (C-3' and C-5'), 129.4 (C-2' and C-6'), 133.5 (C-1'), 139.0 (C-6'''), 140.5 (C-4'''a), 141.2 (C-4'), 159.1 (C-8'''), 162.1 (CONH), 169.8 (C-1'''), 170.5 (C-1); **HRMS** (TOF ESI+) calcd for $C_{29}H_{37}ClNO_7$ $[M+H]^{+}$ 546.2253, found 546.2245.



10 11

***tert*-Butyl (2*S*)-3-(4-(5-azidopentyl)phenyl)-2-(5-chloro-8-hydroxy-3-methyl-1-oxoisochromane-7-carboxamido)propanoate (11).** A solution of PPh_3 (73.3 mg, 0.275 mmol, 1.6 equiv) in anhydrous CH_2Cl_2 (1 mL) was added to a solution of alcohol **10** (94.4 mg, 0.173

mmol) and CBr_4 (120.8 mg, 0.361 mmol, 2.1 equiv) at 0 °C under N_2. The reaction mixture was stirred at rt for 3 h, after which it was diluted with CH_2Cl_2 and washed successively with water and brine. The organic phase was dried over anhydrous $MgSO_4$ and concentrated under reduced pressure to give a residue, which was purified by column chromatography, using hexane-EtOAc mixtures (100:0, 95:5 and 80:20) as eluent, to obtain the corresponding bromide derivative (54.8 mg, 55%) as a yellowish oil (a 1:1 mixture of diastereoisomers). A solution of this bromide intermediate (54.8 mg, 0.09mmol) and NaN_3 (97.5 mg, 1.5 mmol, 16.7 equiv.) in anhydrous DMF (2 mL) was stirred overnight at 35 °C under N_2, after which it was diluted with EtOAc and washed successively with an aqueous solution of LiCl (1.5%) and brine. The organic phase was dried over anhydrous $MgSO_4$ and concentrated under reduced pressure to obtain azide **11** (51 mg, 100%) as a yellowish oil (a 1:1 mixture of diastereoisomers). **^{1}H NMR** ($CDCl_3$, 300 MHz) δ (ppm) 1.36-1.40 (m, 2H, H_2-3''), 1.42 (s, 9H, CMe_3), 1.58-1.68 (m, 7H, H_2-2'', H_2-4'' and Me-3'''), 2.59 (t, *J* = 7.6 Hz, 2H, H_2-1''), 2.85 and 2.86 (two dd, *J* = 17.4, 11.6 Hz, each 0.5H, H-4''' of each diastereoisomer), 3.17 (m, 2H, H_2-3), 3.22-3.34 (m, 3H, H_2-5'' and H-4'''), 4.76 (m, 1H, H-3'''), 4.94 (dt, *J* = 7.4, 6.0 Hz, 1H, H-2), 7.05-7.14 (m, 4H, H-2', H-3', H-5' and H-6'), 8.45 (s, 1H, H-6'''), 8.54 (m, 1H, NH), 12.71 (br s, 1H, OH); **^{13}C NMR** ($CDCl_3$, 75 MHz) δ (ppm) 20.7 (Me-3'''), 26.3 (C-3''), 28.0 (CMe_3), 28.7 (C-4''), 30.9 (C-2''), 32.3 (C-4'''), 35.3 (C-1''), 37.7 (C-3), 51.4 (C-5''), 54.6 (C-2), 75.9 (C-3'''), 82.2 (CMe_3), 110.0 (C-8'''a), 120.9 (C-7'''), 123.0 (C-5'''), 128.4 (C-3' and C-5'), 129.4 (C-2' and C-6'), 133.6 (C-1'), 139.0 (C-6'''), 140.5 (C-4'''a), 140.9 (C-4'), 159.1 (C-8'''), 162.1 (CONH), 169.7 (C-1'''), 170.4 (C-1); **HRMS** (TOF ESI+) calcd for $C_{29}H_{36}ClN_4O_6$ $[M+H]^{+}$ 571.2318, found 571.2300.

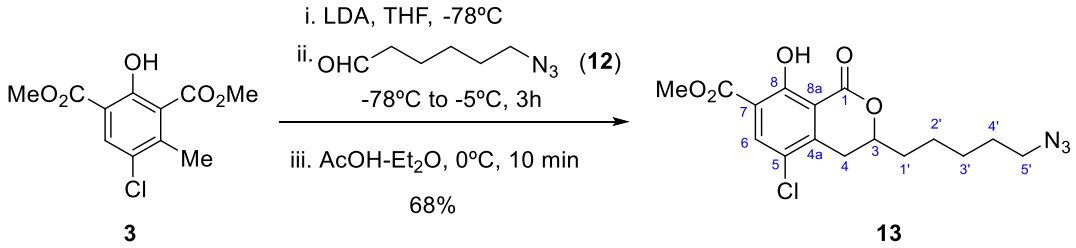


11 hapten **a**

(2*S*)-3-(4-(5-Azidopentyl)phenyl)-2-(5-chloro-8-hydroxy-3-methyl-1-oxoisochromane-7-carboxamido)propanoic acid (hapten **a).** Trifluoroacetic acid (600 µL, 7.8 mmol) was dropwise added to a solution of *tert*-butyl ester **11** (45 mg, 0.078 mmol) in anhydrous CH_2Cl_2 (1.2 mL) and the resulting mixture was stirred for 2 h at rt. Afterwards, the reaction mixture was concentrated to dryness to afford hapten **a** (40.5 mg, nearly quantitative) as a resinous and yellowish product (a 1:1 mixture of diastereoisomers). **^{1}H NMR** ($CDCl_3$, 300 MHz) δ (ppm) 1.35-1.44 (m, 2H, H_2-3''), 1.54-1.67 (m, 7H, H_2-2'', H_2-4'' and Me-3'''), 2.58 (t, *J* = 7.6 Hz, 2H, H_2-

1''), 2.83 and 2.84 (two dd, *J* = 17.0, 11.3 Hz, each 0.5H, H-4''' of each diastereoisomer), 3.12-3.34 (m, 5H, H_2-3, H_2-5'' and H-4'''), 4.74 (m, 1H, H-3'''), 4.97 (m, 1H, H-2), 7.08-7.16 (m, 4H, H-2', H-3', H-5' and H-6'), 8.39 (s, 1H, H-6'''), 8.47 (m, 1H, NH) 12.71 (br s, 1H, OH); **^{13}C NMR** ($CDCl_3$, 75 MHz) δ (ppm) 20.6 (Me-3'''), 26.3 (C-3''), 28.6 (C-4''), 30.8 (C-2''), 32.2 (C-4'''), 35.3 (C-1''), 37.0 (C-3), 51.3 (C-5''), 54.3 (C-2), 75.8 (C-3'''), 109.9 (C-8'''a), 120.6 (C-7'''), 123.0 (C-5'''), 128.4 (C-3' and C-5'), 129.4 (C-2' and C-6'), 133.5 (C-1'), 138.9 (C-6'''), 141.6 (C-4a'''), 140.9 (C-4'), 159.0 (C-8'''), 162.6 (CONH), 169.7 (C-1'''), 173.5 (C-1); **HRMS** (TOF ESI+) calcd for $C_{25}H_{28}ClN_4O_6$ $[M+H]^{+}$ 515.1692, found 515.1686.

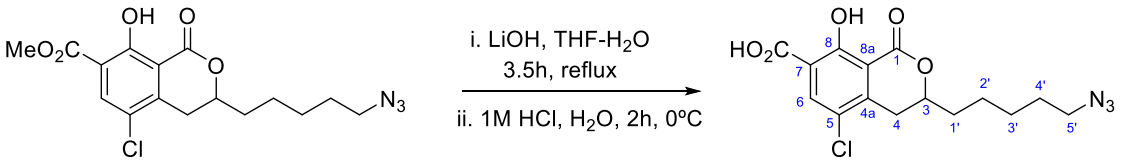
Synthesis of hapten c



Methyl 3-(5-azidopentyl)-5-chloro-8-hydroxy-1-oxoisochromane-7-carboxylate (13). A solution of diester **3** (366.0 mg, 1.42 mmol) in anhydrous THF (1.5 mL) was added dropwise to a solution of LDA in THF [generated from diisopropylamine (580 µL, 3.9 mmol, 2.75 equiv) and 1.6 M BuLi in hexane (2.2 mL, 3.55 mmol, 2.5 equiv) in THF (4 mL)] at –78 °C under nitrogen. The mixture was maintained at the same temperature for 30 min and then a solution of aldehyde **12**[4] (320 mg, 2.2 mmol, 1.6 equiv) in anhydrous THF (500 µL) was added. After stirring at –78 °C for 15 min and warming slowly to 5 °C (about 3 hours), the reaction mixture was treated with a 1:2 mixture of AcOH-Et_2O and stirred for 10 minutes at 0 °C. Afterwards, the reaction mixture was diluted with EtOAc and washed with water and brine and dried over anhydrous $MgSO_4$. Chromatographic purification of the residue obtained by evaporation of the solvent under reduced pressure, using hexane-EtOAc-AcOH mixtures (100:0:0.3, 95:5:0.3 and 85:15:0.3) as eluent, afforded compound **13** (356.0 mg, 68%) as a yellowish oil. **^{1}H NMR** ($CDCl_3$, 300 MHz) δ (ppm) 1.35-2.00 (m, 8H, H_2-1', H_2-2', H_2-3' and H_2-4'), 2.85 (dd, *J* = 17.3, 11.7 Hz, 1H, H-4), 3.24 (dd, *J* = 17.3, 3.2 Hz,, 1H, H'-4), 3.30 (t, *J* = 6.7 Hz, 2H, H_2-5'), 3.94 (s, 3H, OMe), 4.56 (m, 1H, H-3), 8.09 (s, 1H, H-6), 12.16 (s, 1H, OH); **^{13}C NMR** ($CDCl_3$, 75 MHz) δ (ppm) 24.3

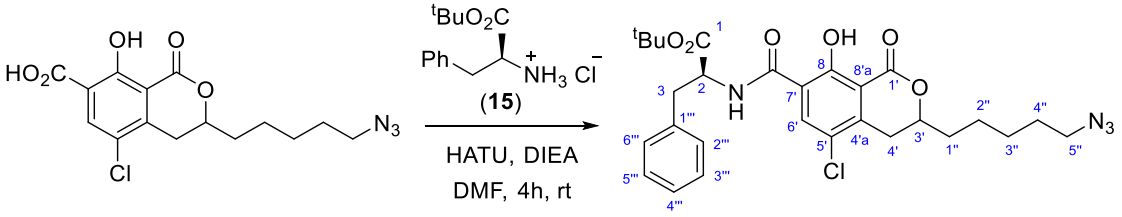
[4] (a) M.K. Malkoch, Schleicher, E.D rockenmuller, C. J. Hawker, T. P. Russell, P. Wu and V. V. Fokin. Structurally diverse dendritic libraries: A highly efficient functionalization approach using click chemistry. *Macromolecules*, 2005, **38**, 3663–3678. (b) T. M. Shaikh and A. Sudalai. Enantioselective Synthesis of (+)-α-Conhydrine and (-)-Sedamine by L-ProlineCatalysed α-Aminooxylation. European *J. Org. Chem*., 2010, 3437–3444.

(C-2'), 26.4 (C-3'), 28.6 (C-4'), 31.0 (C-4), 34.5 (C-1'), 51.2 (C-5'), 52.6 (OMe), 78.4 (C-3), 111.4 (C-8a), 118.3 (C-7), 121.8 (C-5), 138.0 (C-6), 142.2 (C-4a), 161.0 (C-8), 165.0 (C-1), 167.7 (CO_2); **HRMS** (TOF ESI+) calcd for $C_{16}H_{19}ClN_3O_5$ $[M+H]^{+}$ 368.1008, found 368.1020.



13 90% **14**

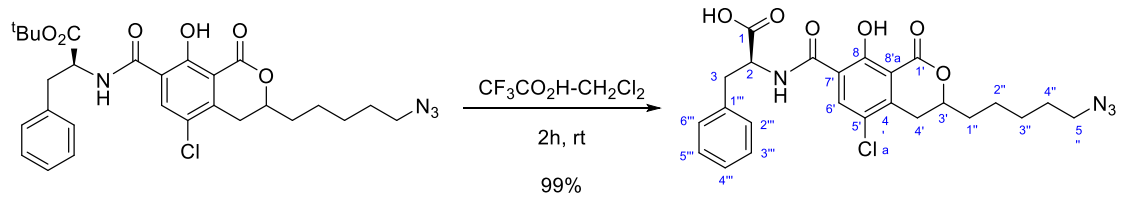
3-(5-Azidopentyl)-5-chloro-8-hydroxy-1-oxoisochromane-7-carboxylic acid (14). A solution of $LiOH{\cdot}H_2O$ (398.0 mg, 9.5 mmol, 10 equiv) in water (4.0 mL) was added to a solution of dihydroisocoumarin **13** (350.0 mg, 0.95 mmol) in anhydrous THF (3.5 mL) and the mixture was heated at reflux for 3.5 h. Afterwards, the reaction mixture was cooled in an ice bath, treated with a 1 M aqueous solution of HCl (17 mL, 17.1 mmol, 18 equiv), stirred for 2 h at 0 °C, diluted with water and extracted with EtOAc. The combined organic phases were washed with brine, dried over anhydrous $MgSO_4$ and concentrated to dryness under reduced pressure to obtain acid **14** (301.0 mg, 90%) as a yellowish semi-solid whose ^{1}H NMR spectrum showed to have a sufficiently high purity to be used in the next stage without further purification. **^{1}H NMR** ($CDCl_3$, 300 MHz) δ (ppm) 1.43-1.76 (m, 6H, H_2-1', H_2-2' and H_2-3'), 1.78-2.05 (m, 2H, H_2-4'), 2.93 (dd, *J* = 17.7, 11.7 Hz, 1H, H-4), 3.27-3.36 (m, 3H, H_2-5' and H'-4), 4.62-4.72 (m, 1H, H-3), 8.40 (s, 1H, H-6), 13.21 (s, 1H, OH); **^{13}C NMR** ($CDCl_3$, 75 MHz) δ (ppm) 24.3 (C-2'), 26.4 (C-3'), 28.7 (C-4'), 30.7 (C-4), 34.5 (C-1'), 51.2 (C-5'), 79.5 (C-3), 110.6 (C-8a), 117.3 (C-7), 124.0 (C-5), 139.8 (C-6), 143.0 (C-4a), 159.2 (C-8), 163.5 (C-1), 169.3 (CO_2H); **HRMS** (TOF ESI+) calcd for $C_{15}H_{17}ClN_3O_5$ $[M+H]^{+}$ 354.0851, found 354.0841.



14 76% **16**

***tert*-Butyl (3-(5-azidopentyl)-5-chloro-8-hydroxy-1-oxoisochromane-7-carbonyl)-*L*-phenylalaninate (16).** A solution of HATU (482.0 mg, 1.27 mmol, 1.5 equiv) in anhydrous DMF (3 mL) and DIEA (295 µL, 1.7 mmol, 2 equiv) was added to a solution of acid **14** (300.0 mg, 0.85 mmol) in anhydrous DMF (3 mL) under nitrogen and the resulting mixture was stirred at rt for 2 h. Next, a solution of hydrochloride **15** (416.0 mg, 1.70 mmol, 2 equiv) and DIEA (295 µL, 1.7

mmol, 2 equiv) in anhydrous DMF (3 mL) was added and the mixture stirred at the same temperature for 4 h. The reaction mixture was diluted with EtOAc and washed successively with aqueous solutions of HCl (1 M), LiCl (1.5%), $NaHCO_3$ (5%) and brine, dried over anhydrous $MgSO_4$ and concentrated to dryness under reduced pressure. The residue obtained was purified by chromatography on silica gel, using hexane-EtOAc-AcOH mixtures (100:0:0.3 and 90:10:0.3) as eluent, to obtain amide **16** (359.0 mg, 76%) as a yellowish oil (a 1:1 mixture of diastereoisomers). **^{1}H NMR** ($CDCl_3$, 300 MHz) δ (ppm) 1.37-1.95 (m, 8H, H_2-1'', H_2-2'', H_2-3'' and H_2-4''), 1.42 (s, 9H, CMe_3), 2.86 and 2.88 (two dd, *J* = 17.3, 11.7 Hz, each 0.5H, H-4' of each diastereoisomer), 3.18-3.25 (m, 3H, H'-4' and H_2-3), 3.31 (t, *J* = 6.7 Hz, 2H, H_2-5''), 4.54-4.67 (m, 1H, H-3'), 4.97 (dt, *J* = 7.3, 6.0 Hz, 1H, H-2), 7.19-7.34 (m, 5H, Ph), 8.46 (s, 1H, H-6'), 8.55 and 8.56 (two br d, *J* = 7.2, each 0.5H, NH of each diastereoisomer); **^{13}C NMR** ($CDCl_3$, 75 MHz) δ (ppm) 24.3 (C-2''), 26.4 (C-3''), 27.9 (*CMe*$_3$), 28.7 (C-4''), 30.7 (C-4'), 34.6 (C-1''), 38.1 (C-3), 51.2 (C-5''), 54.6 (C-2), 79.2 (C-3'), 82.3 (*C*Me_3), 110.1 (C-8'a), 120.9 (C-7'), 123.1 (C-5'), 126.9 (C-4'''), 128.3 (C-2''' and C-6'''), 129.5 (C-3''' and C-5'''), 136.3 (C-1'''), 139.0 (C-6'), 140.5 (C-4'a), 159.0 (C-8'), 162.1 (CONH), 169.7 (C-1'), 170.4 (C-1); **HRMS** (TOF ESI+) calcd for $C_{28}H_{34}ClN_4O_6$ $[M+H]^{+}$ 557.2161, found 557.2162.



16

(3-(5-Azidopentyl)-5-chloro-8-hydroxy-1-oxoisochromane-7-carbonyl)-*L*-phenylalanine (hapten **c).** Trifluoroacetic acid (4.0 mL, 52.0 mmol) was dropwise added to a solution of *tert*-butyl ester **16** (279.0 mg, 0.50 mmol) in anhydrous CH_2Cl_2 (8.0 mL) and the resulting mixture was stirred for 2 h at rt. Afterwards, the reaction mixture was concentrated to dryness to afford hapten **c** (249.8 mg, nearly quantitative) as a resinous and yellowish product (a 1:1 mixture of diastereoisomers). **^{1}H NMR** ($CDCl_3$, 300 MHz) δ (ppm) 1.42-2.00 (m, 8H, H_2-1'', H_2-2'', H_2-3'' and H_2-4''), 2.86 and 2.88 (two dd, *J* = 17.5, 11.7 Hz, each 0.5H, H-4' of each diastereoisomer), 3.15-3.40 (m, 5H, H_2-3, H'-4' and H_2-5''), 4.60 (m, 1H, H-3'), 5.03 (m, 1H, H-2), 7.15-7.37 (m, 5H, Ph), 8.42 (s, 1H, H-6'), 8.49 and 8.50 (two br d, *J* = 7.1, each 0.5H, NH of each diastereoisomer), 12.72 (s, 1H, OH); **^{13}C NMR** ($CDCl_3$, 75 MHz) δ (ppm) 24.3 (C-2''), 26.4 (C-3''), 28.6 (C-4''), 30.6 (C-4'), 34.5 (C-1''), 37.3 (C-3), 51.2 (C-5''), 54.3 (C-2), 79.2 (C-3'), 110.2 (C-8'a), 120.1 (C-7'), 123.2 (C-5'), 127.2 (C-4'''), 128.6 (C-2''' and C-6'''), 129.3 (C-3''' and C-5'''),

135.7 (C-1'''), 138.8 (C-6'), 141.0 (C-4'a), 159.0 (C-8'), 163.0 (CONH), 169.6 (C-1'), 175.1 (C-1); **HRMS** (TOF ESI+) calcd for $C_{24}H_{26}ClN_4O_6$ $[M+H]^+$ 501.1535, found 501.1522.

Preparation of click chemistry-assisted bioconjugates.

Modification of proteins with propargyl groups. Compound **17** was dissolved in DMF and drop wise added to a solution of protein in phosphate buffer (PB, pH 7.4). The conjugation reaction was carried out with a DMF maximum content of 10% (v/v). After stirring overnight at room temperature, the protein conjugates were purified by size-exclusion chromatography using PB as eluent at a flow rate of 5 mL/min.

Table S1. Conditions for the preparation of alkyne-modified proteins

	Protein			Alkyne (17)			
	V (µL)	C (mg/mL)	n (µmol)	V (µL)	C (mM)	n (µmol)	MR_0 [a]
BSA	1800	11.1	0.30	30	250	7.6	25
OVA	1800	15	0.61	100	75	7.5	12
HRP	1700	3	0.12	76	15	1.2	10

[a] Mols of compound **17** added per mol of protein.

Coupling of haptens to alkyne-modified proteins by CuAAC reaction: Solutions of haptens **a** and **c** in DMSO were drop wise added to alkyne-modified protein solutions in PB, pH 7.4. After adding a premixed solution of THPTA/Cu(I) complex (5:1) in MiliQ H_2O, the mixture was purged by repeated vacuum–nitrogen cycles. Finally, a solution of sodium ascorbate in MiliQ H_2O was added and the final reaction volume was adjusted with PB, pH 7.4, followed by vacuum–nitrogen cycles. The solutions were incubated 20 h at room temperature, and the reaction was then stopped using Amicon® Ultra 4 10K centrifugal filters. The protein conjugates were purified by size-exclusion chromatography.

Table S2. CuAAC reaction conditions for each of the alkyne-modified proteins.

					CuAAC Reagents								
	Protein				Hapten		$CuSO_4$		THPTA		Sodium ascorbate		
	N° Alkyne[a]	MW[b]	m (mg)[c]	n_{alkyne} (µmol)[d]	C[e]	Equiv[f]	C	Equiv	C	Equiv	C	Equiv	V_{final} (mL)[g]
BSA–Alkyne	17	68.5	12	3.06	20	6	45	0.4	115	2	100	20	30
OVA–Alkyne	7	46.1	5	0.77	10	2	45	0.2	115	1	100	10	8
HRP–Alkyne	1	44.1	1	0.02	10	4	4.5	0.2	1.2	1	10	10	1

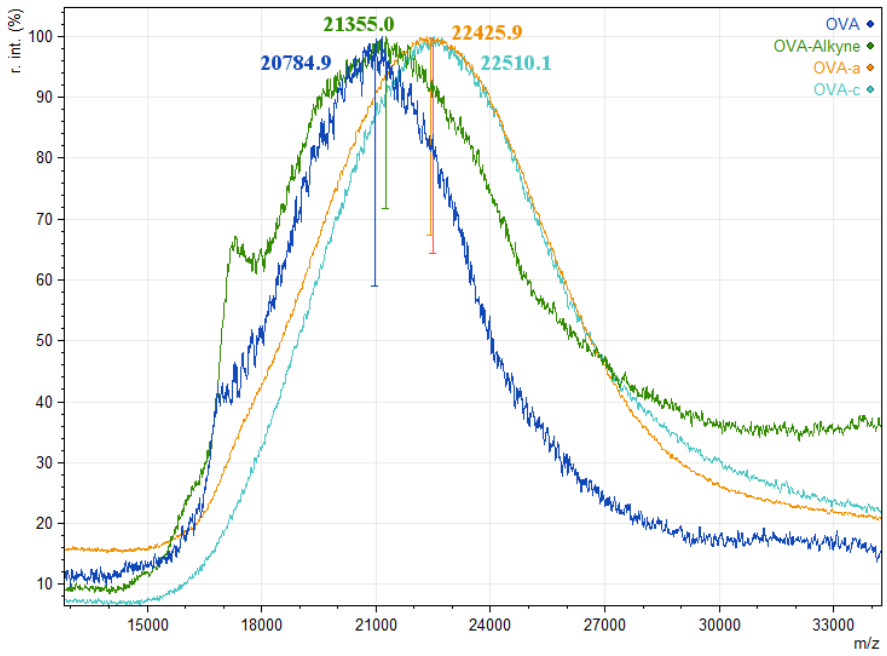
[a] Number of alkyne groups per protein. [b] Estimated molecular weight of the alkyne-modified proteins by MALDI-TOF analysis (kDa). [c] Mass of alkyne-modified protein employed in the CuAAC reaction. [d] Mols of alkyne present during the CuAAC reaction. [e] Concentration of freshly prepared solutions (mM). [f] Mols of hapten per mol of propargyl groups in the protein. [g] Final reaction volume.

MALDI-TOF mass spectrometry analysis of modified proteins

Sample preparation. 100 µL of native protein, alkyne-modified protein or protein–hapten bioconjugate (0.5–1 mg/mL) was dialyzed against MilliQ water with Slide-A-Lyzer® MINI dialysis units and then freeze-dried and lyophilized. The samples were dissolved in Milli-Q H_2O to a final concentration of 1 µg/µL. Then, 1 µL of every sample solution was spotted onto the MALDI plate. After the droplets were air dried at room temperature, 1 µL of matrix [10 mg/mL sinapinic acid in 0.1% trifluoroacetic acid–CH_3CN/H_2O (7:3 v/v)] was added and allowed to air-dry at room temperature.

Mass spectrometry analysis. The resulting mixtures were analysed in a 5800 MALDI TOF/TOF (ABSciex) in positive linear mode (1500 shots every position) in a mass range of 15000–100000 *m/z*. Previously, the plate was calibrated with 1 µL of the TOF/TOF calibration mixture (ABSciex), in 13 positions. Every sample was calibrated by 'close external calibration' method with a BSA, OVA, or HRP spectrum acquired in a close position.

(A)



(B)

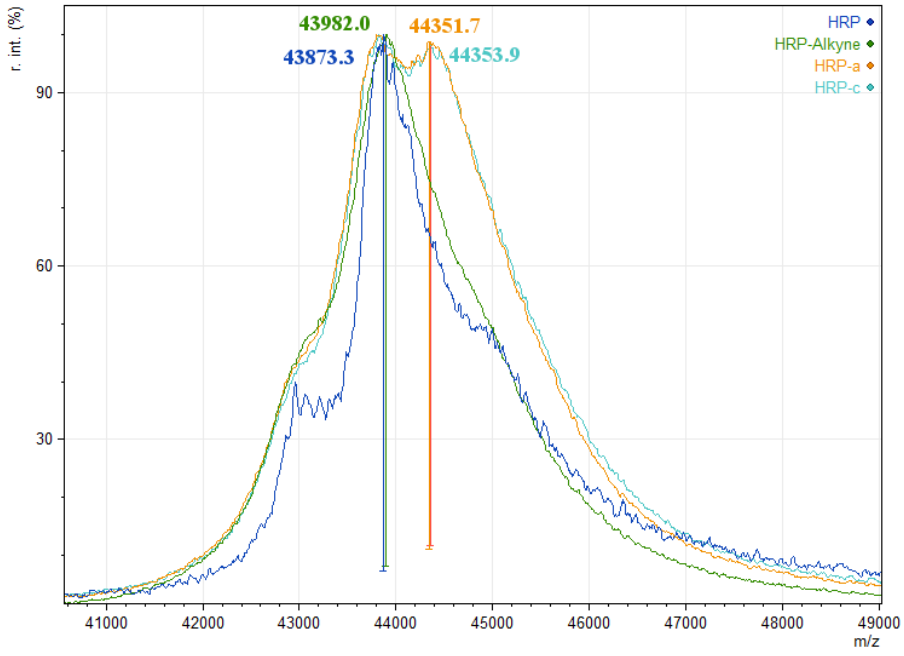
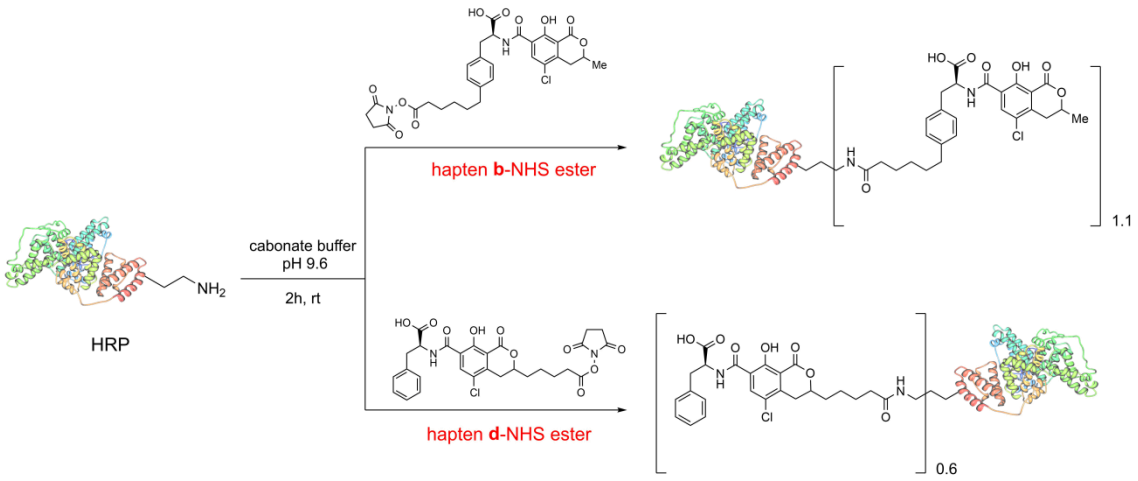
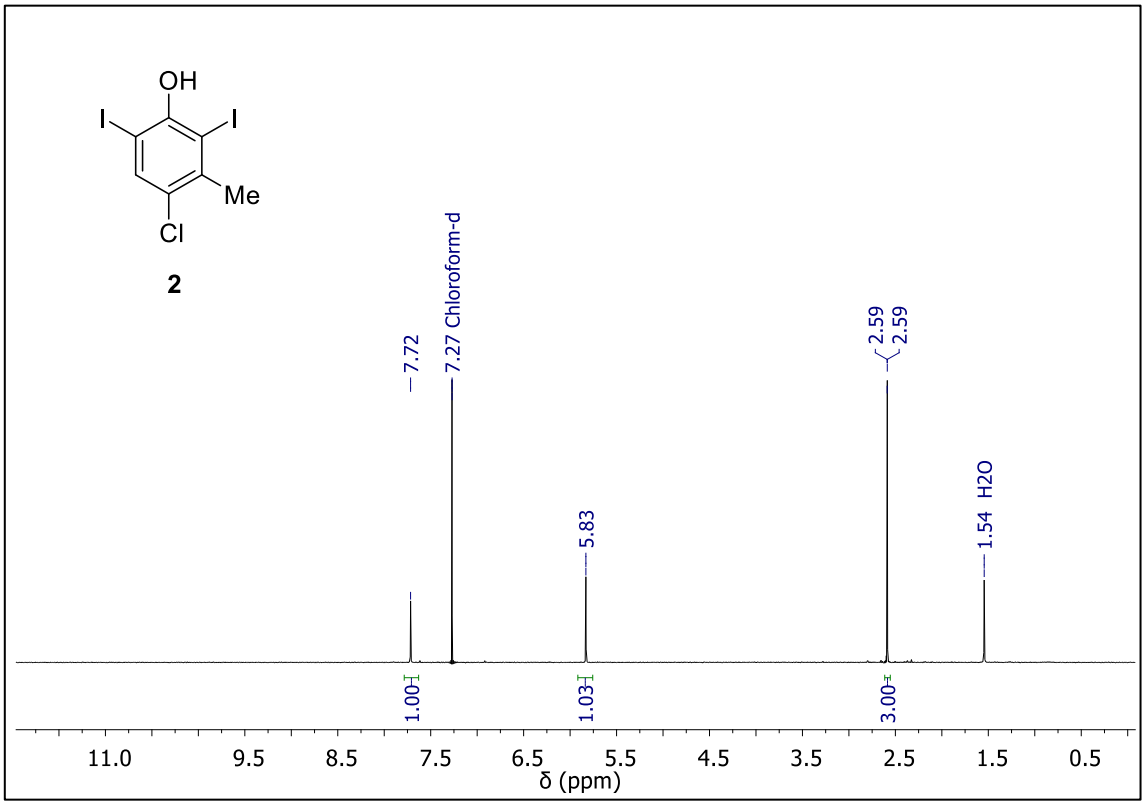


Figure S1. MALDI-TOF mass spectra of OVA (A, doubly charged ions) and HRP (B, singly charged ions) in their unmodified form (blue), modified with propargyl groups (green), and when coupled to haptens **a** (orange) and **c** (light blue).

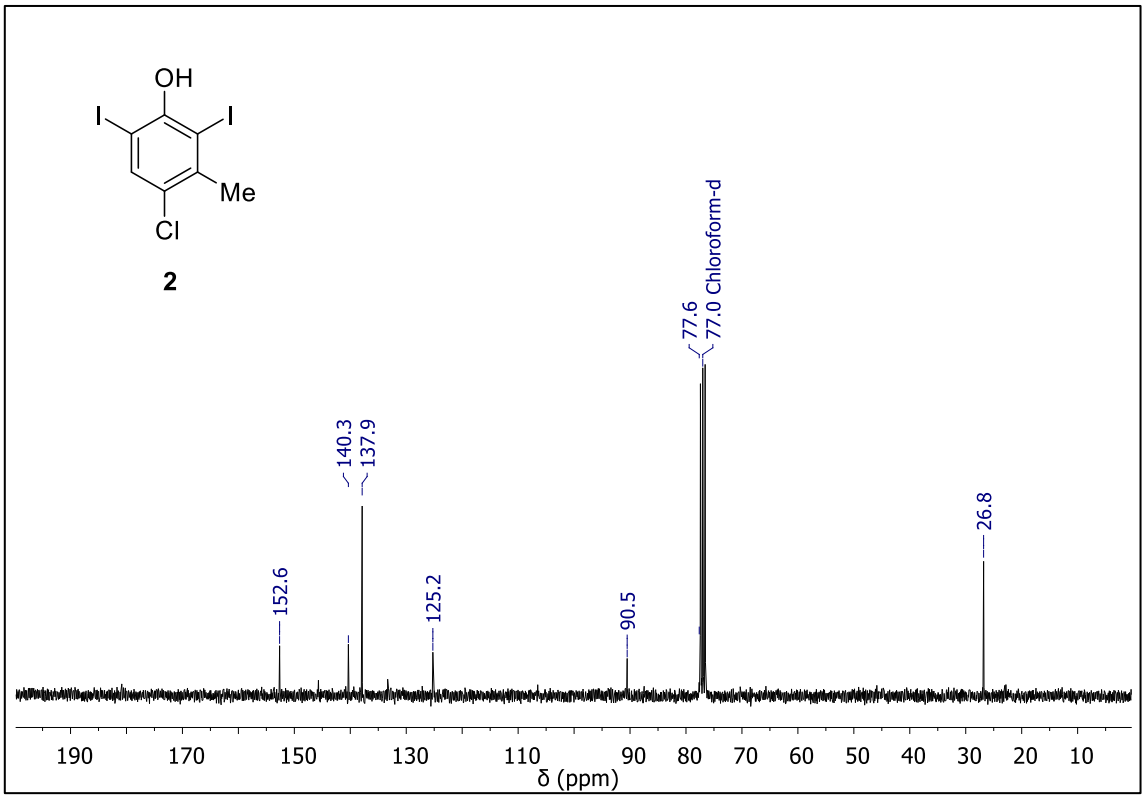


Scheme S1. Coupling of haptens **b** and **d**, as *N*-hydroxysuccinimyl esters, to HRP. Please note that the linker attachment site in hapten **b** is the same than in hapten **a**. Likewise, the handle position in hapten **d** is identical to that in hapten **c**.

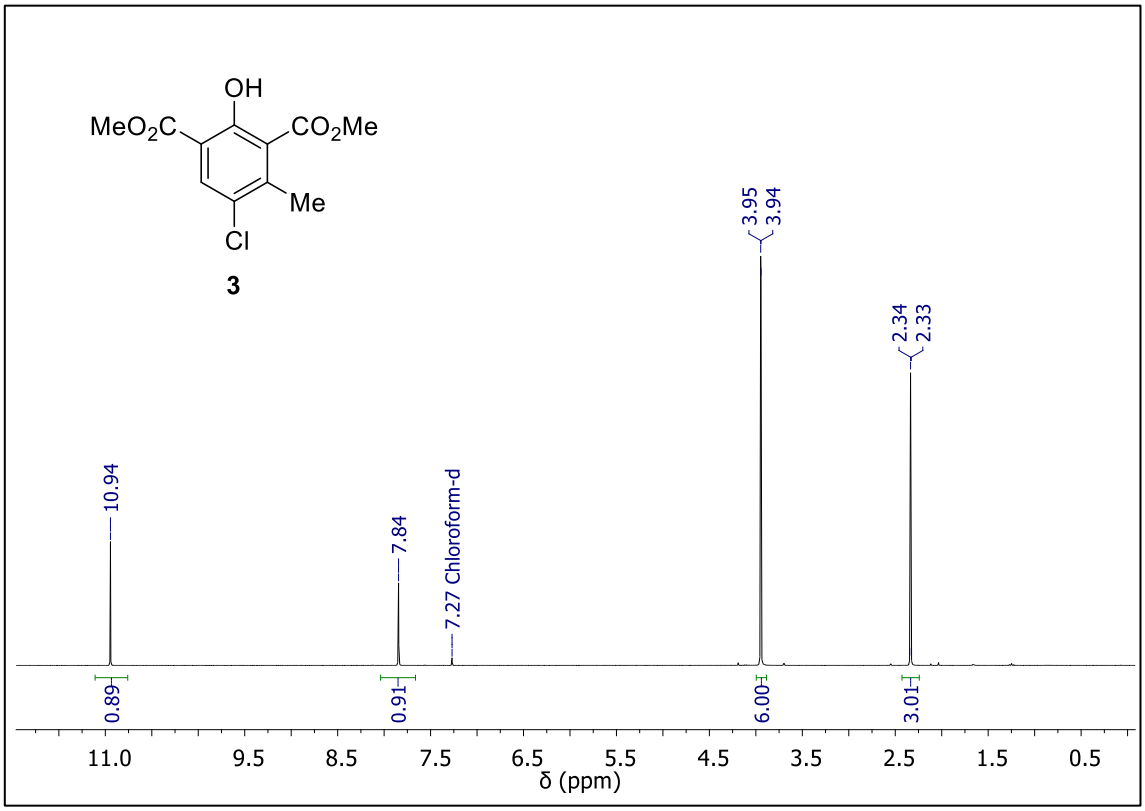
^{1}H-NMR spectrum of intermediate **2**.



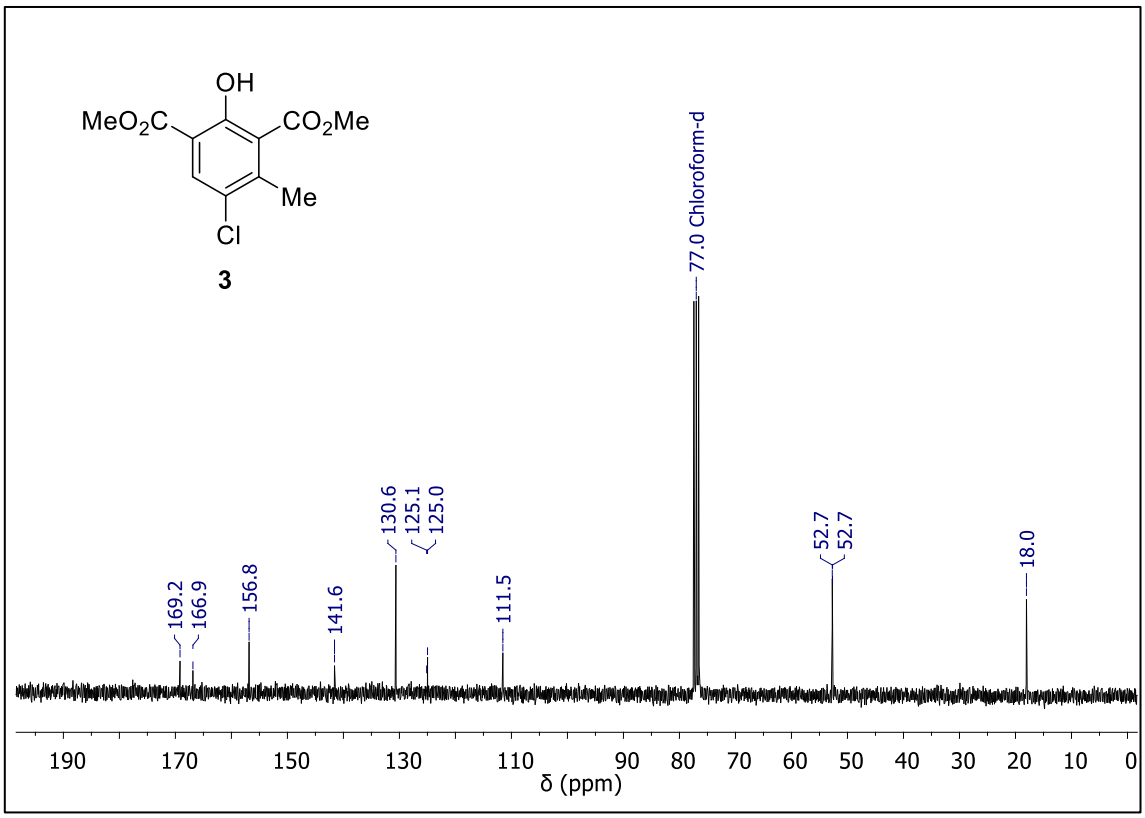
^{13}C-NMR spectrum of intermediate **2**.



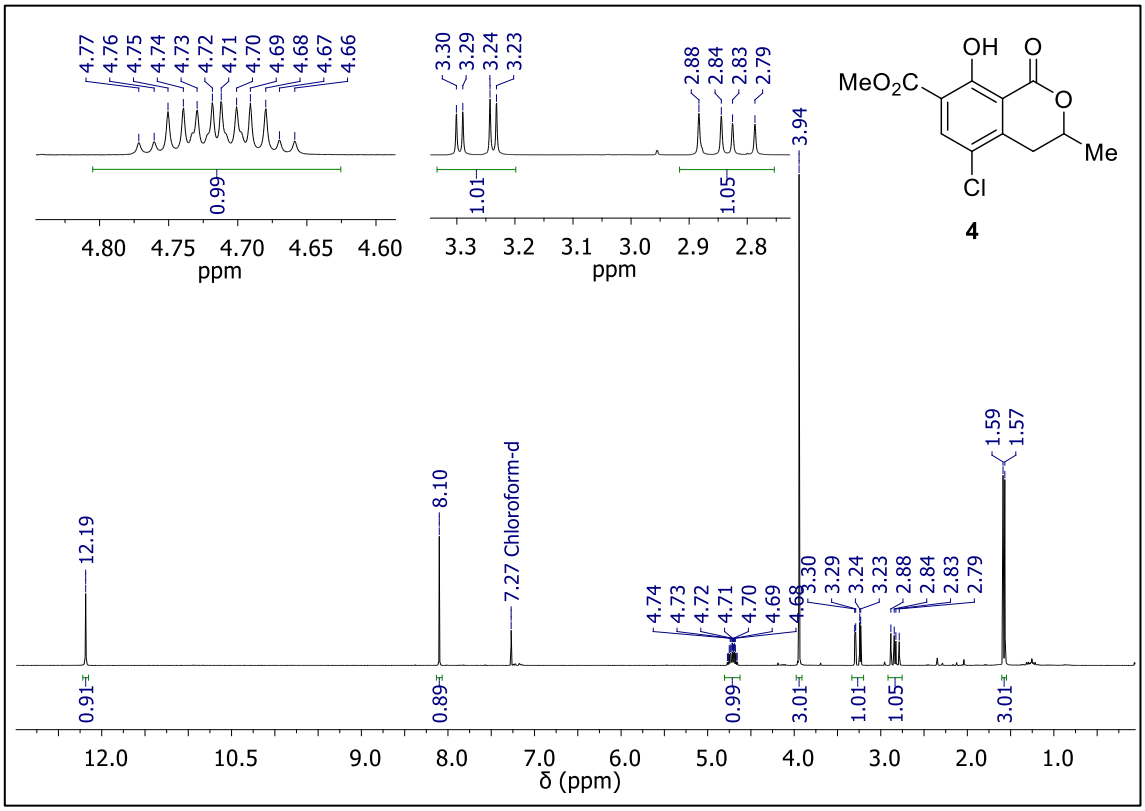
^{1}H-NMR spectrum of intermediate **3**.



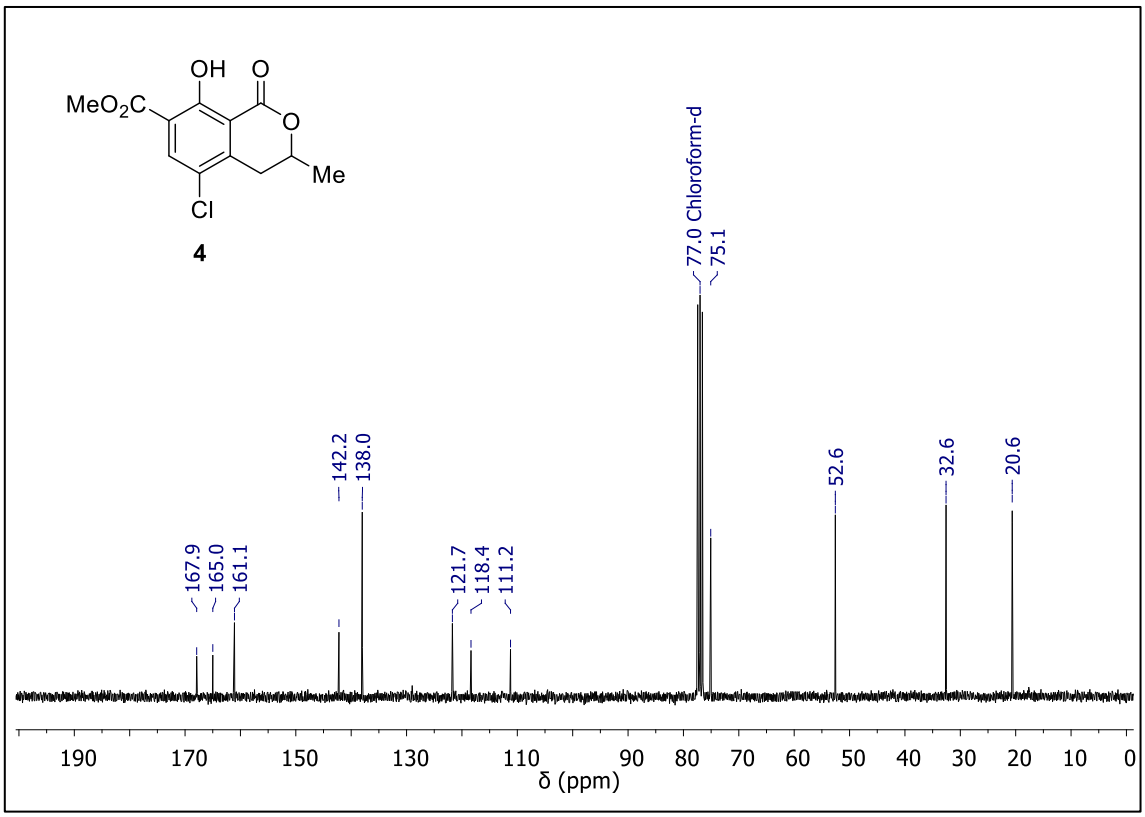
^{13}C-NMR spectrum of intermediate **3**.



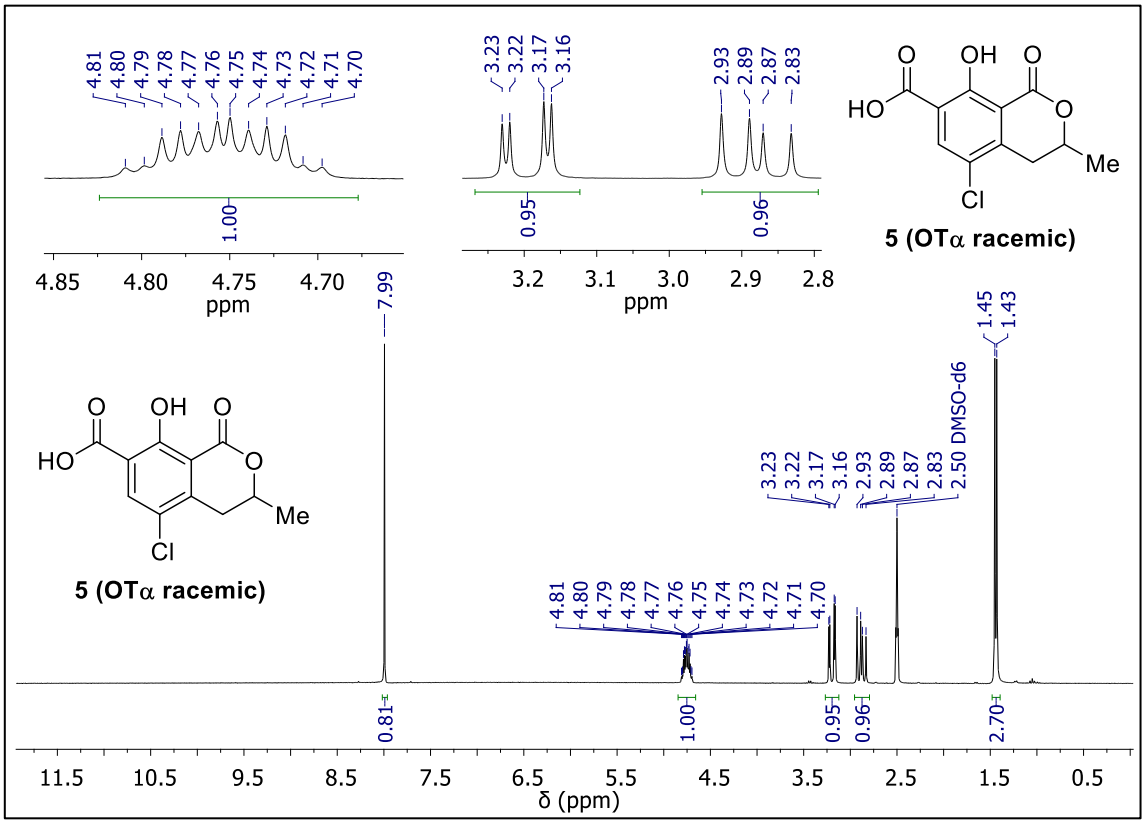
^{1}H-NMR spectrum of intermediate **4**.



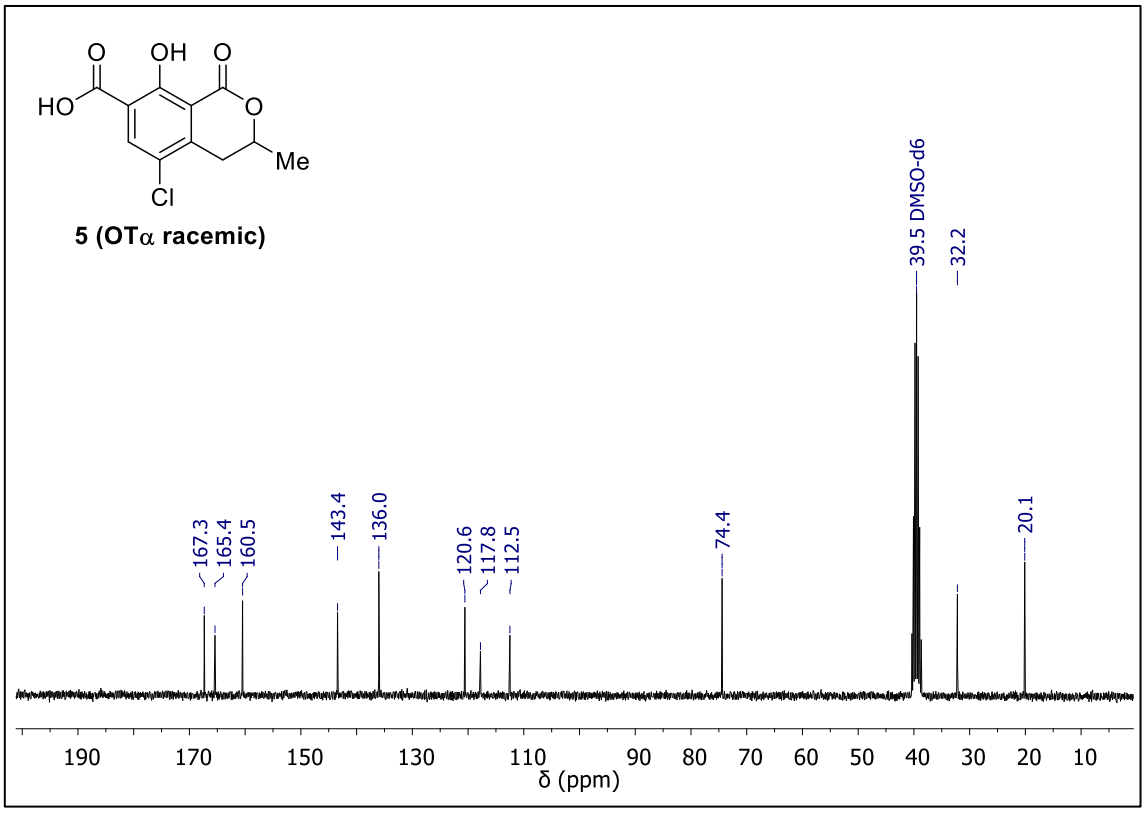
^{13}C-NMR spectrum of intermediate **4**.



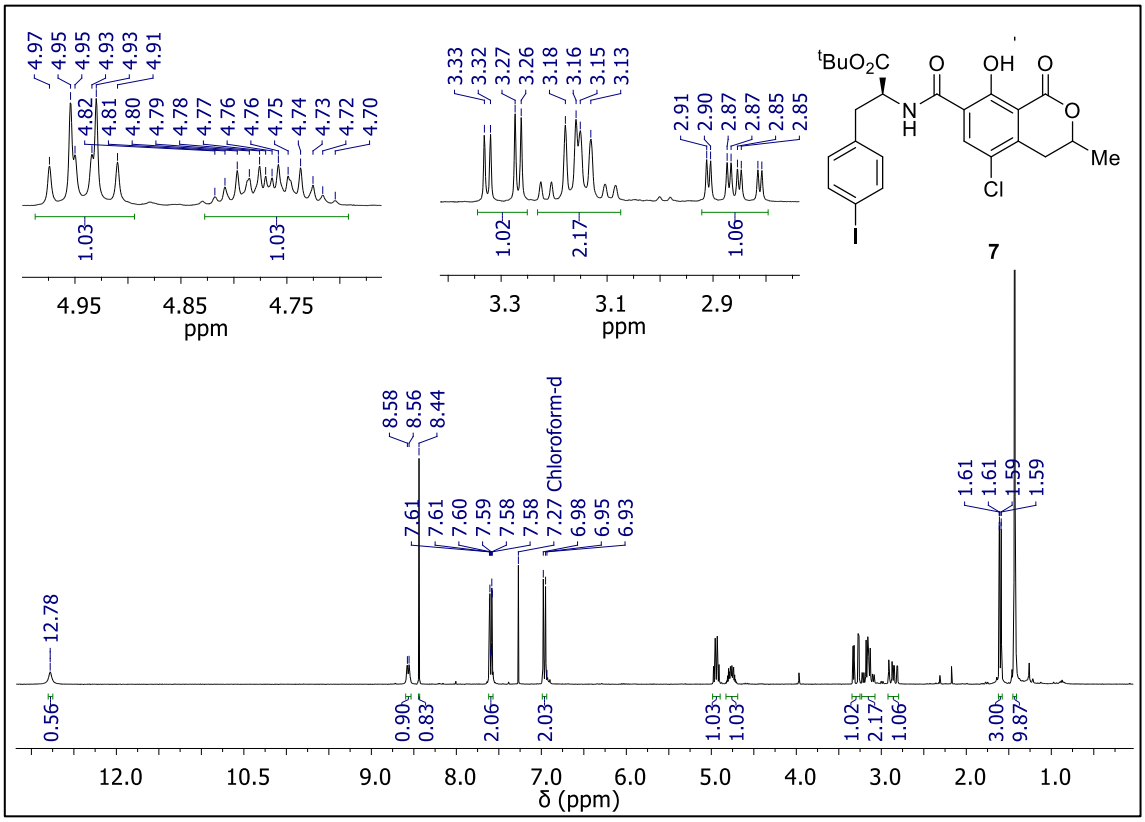
^{1}H-NMR spectrum of intermediate **5**.



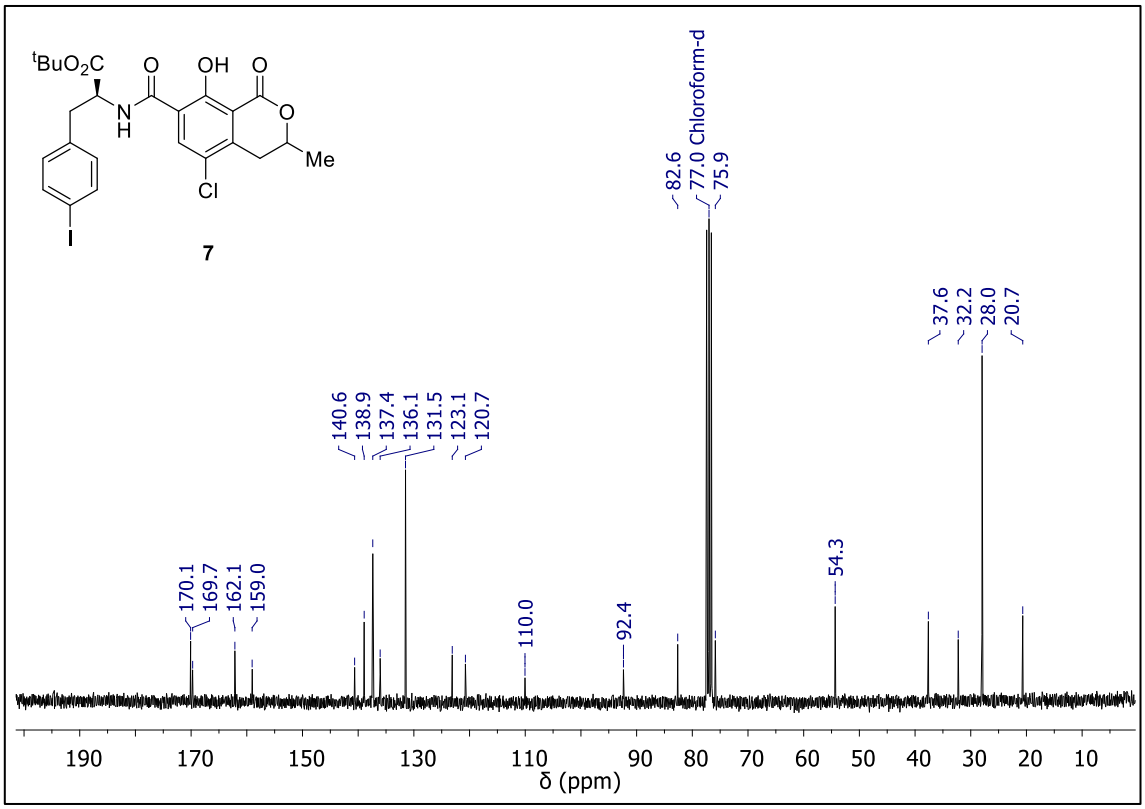
^{13}C-NMR spectrum of intermediate **5**.



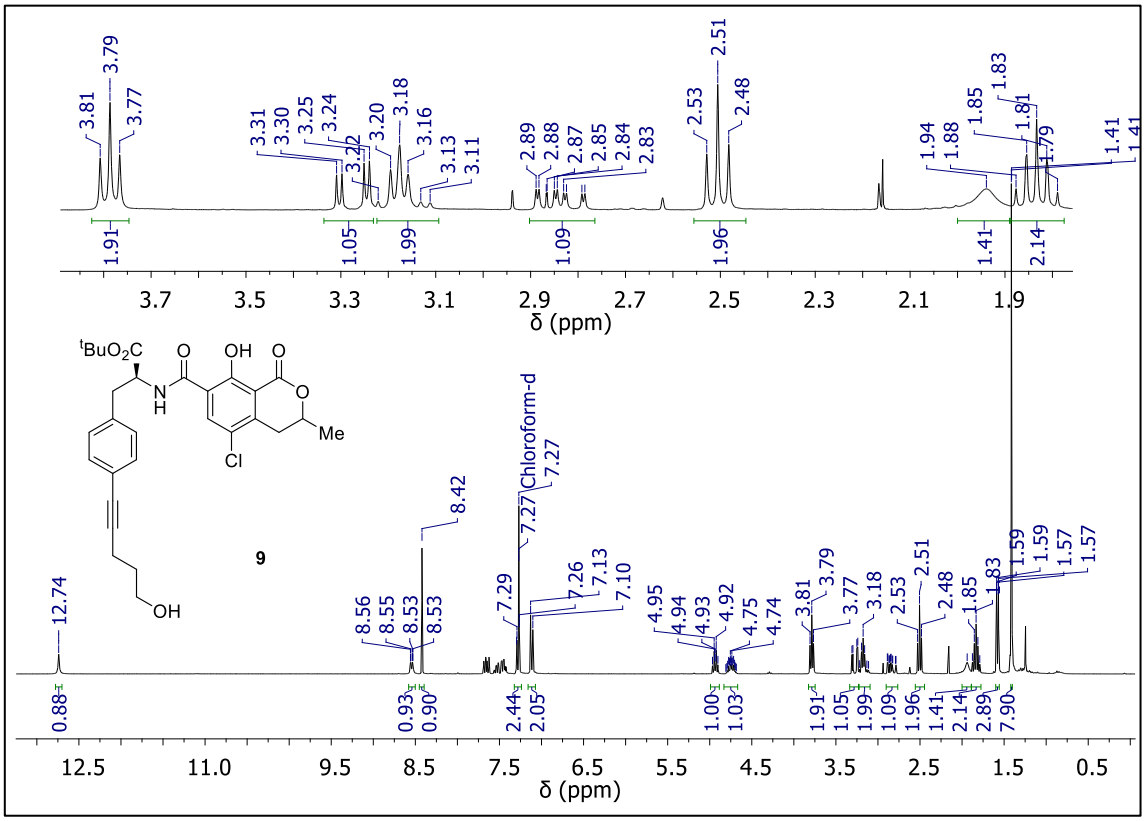
^{1}H-NMR spectrum of intermediate **7**.



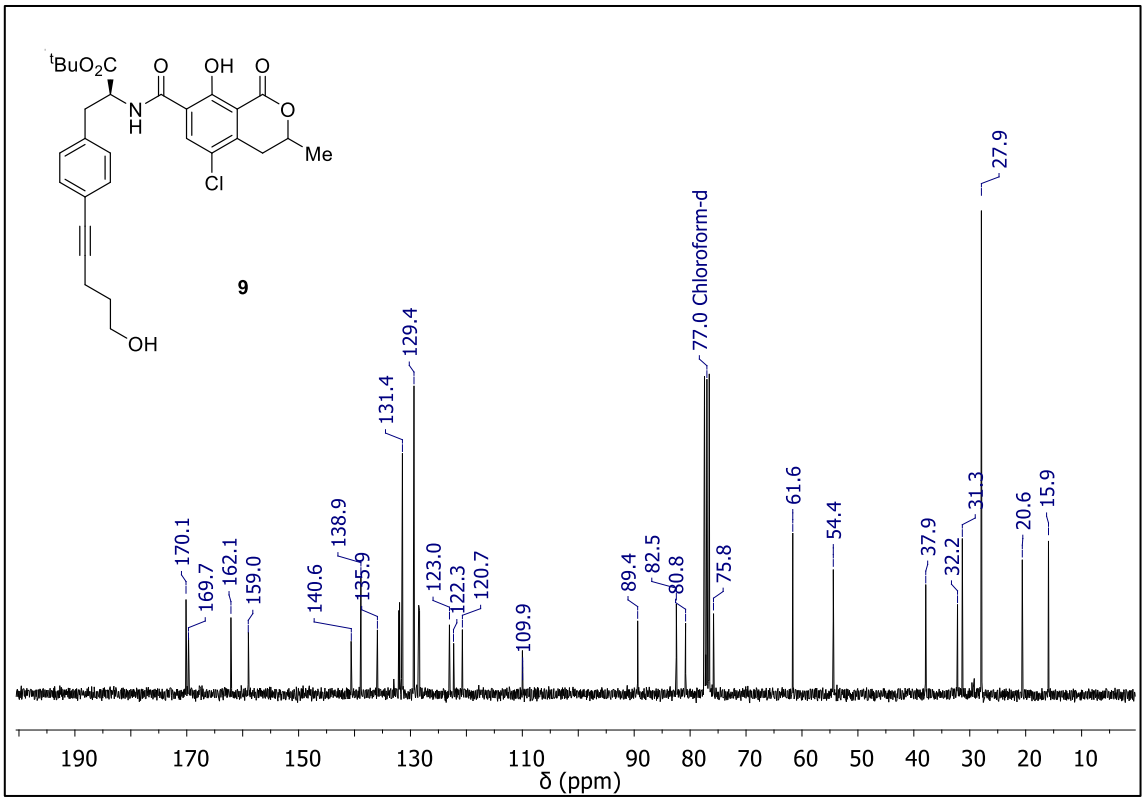
^{13}C-NMR spectrum of intermediate **7**.



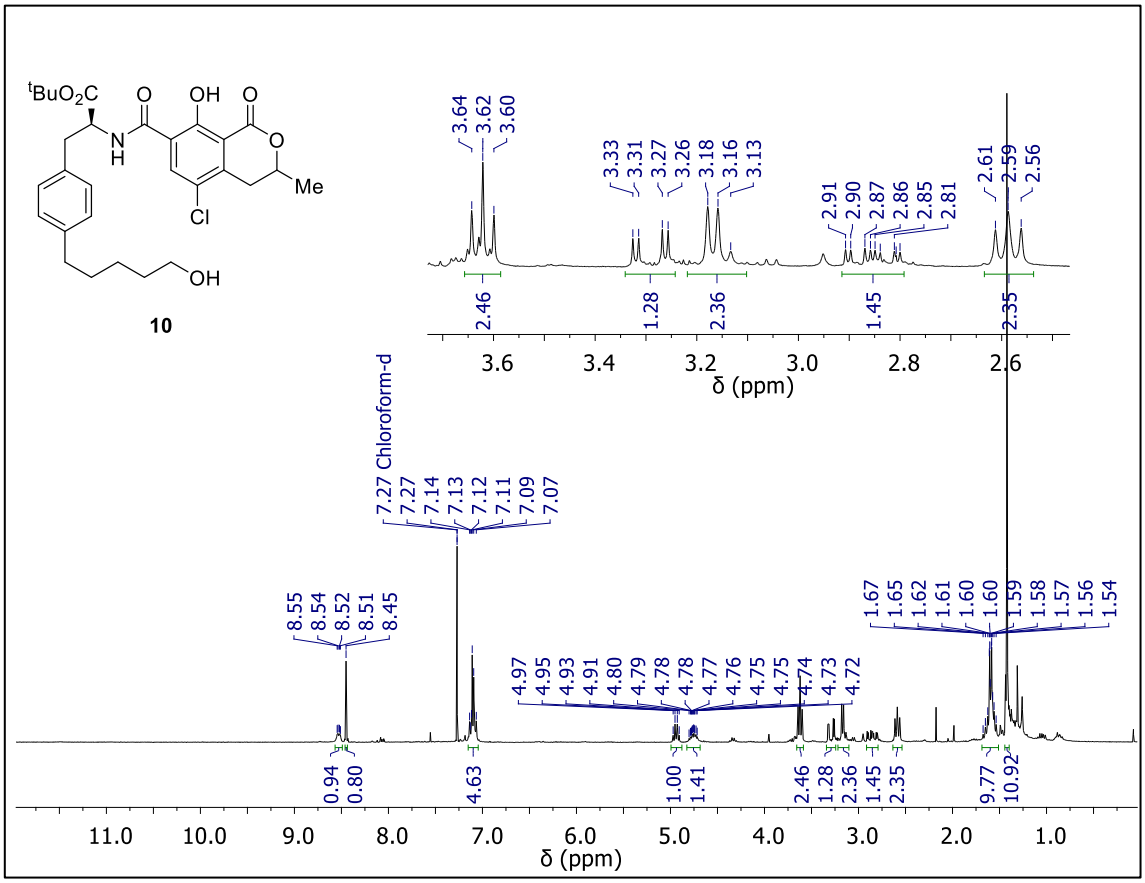
^{1}H-NMR spectrum of intermediate **9**.



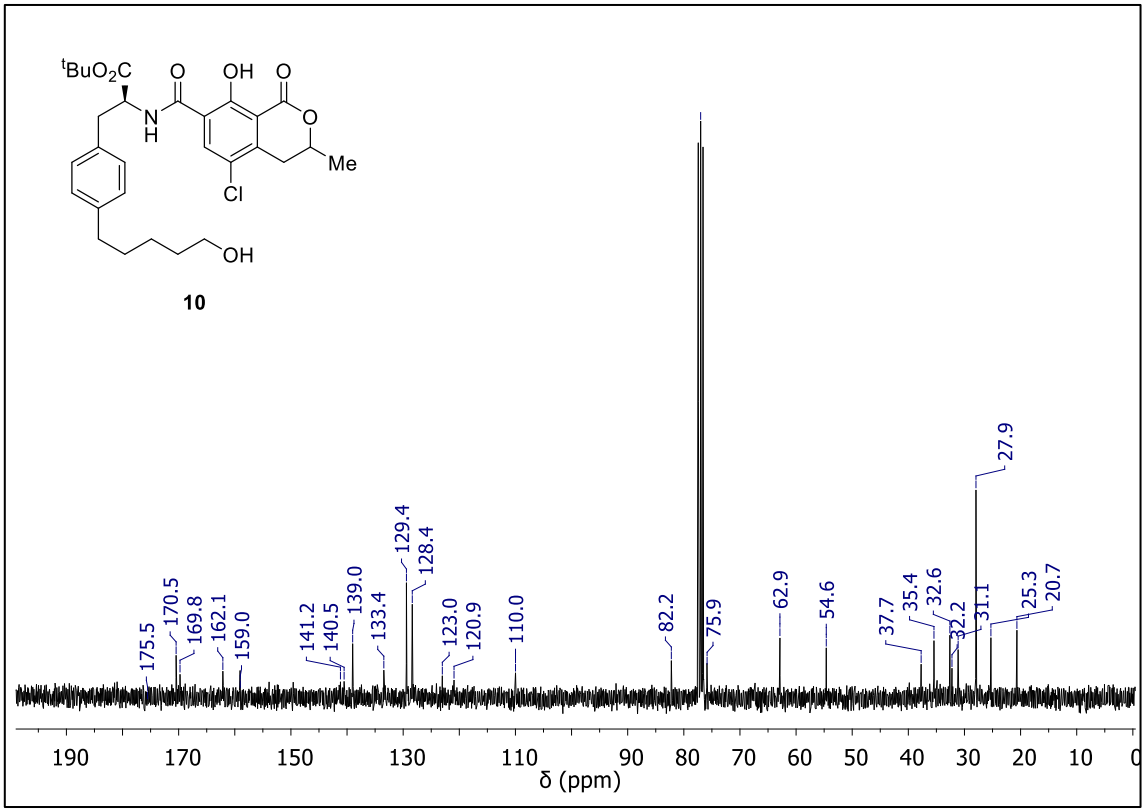
^{13}C-NMR spectrum of intermediate **9**.



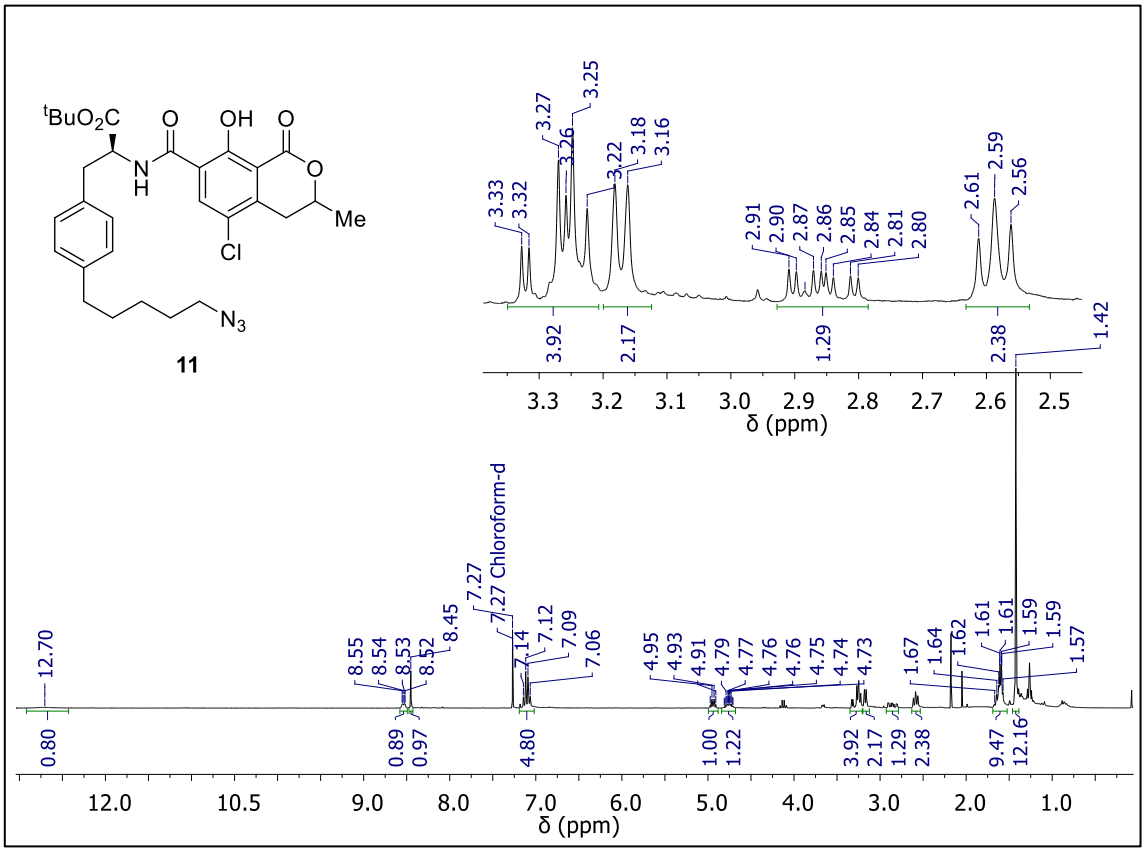
^{1}H-NMR spectrum of intermediate **10**.



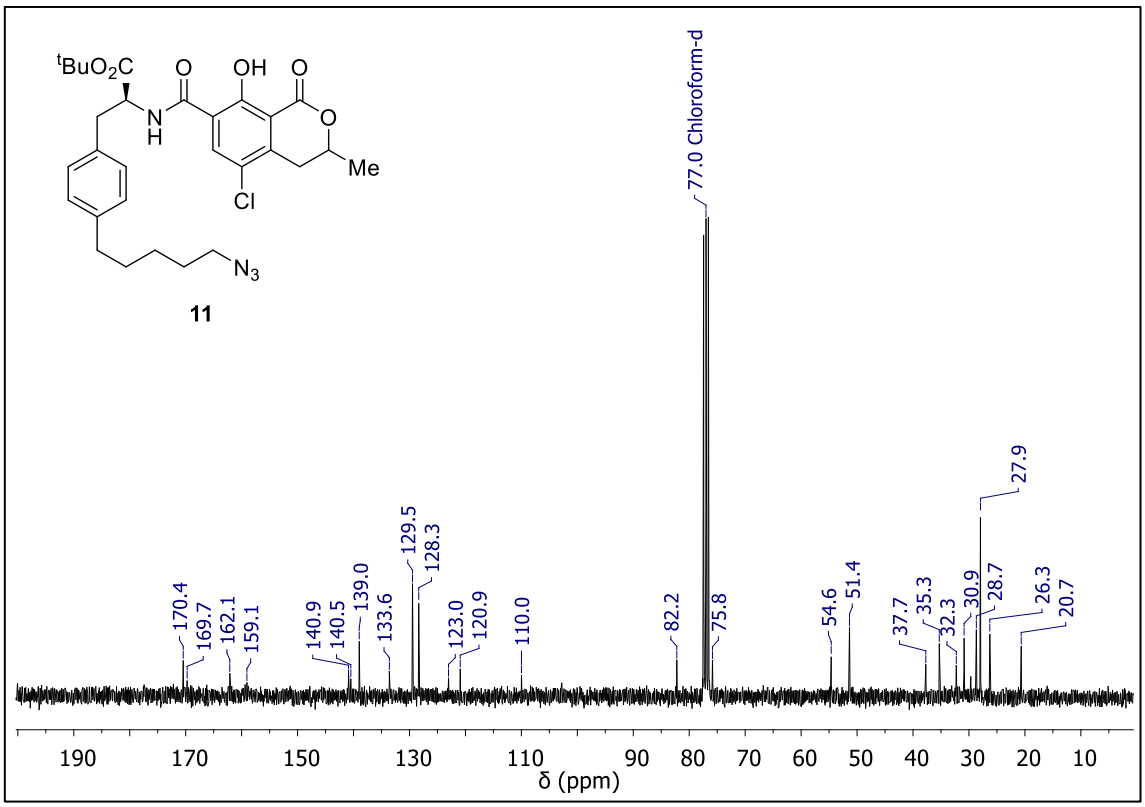
^{13}C-NMR spectrum of intermediate **10**.



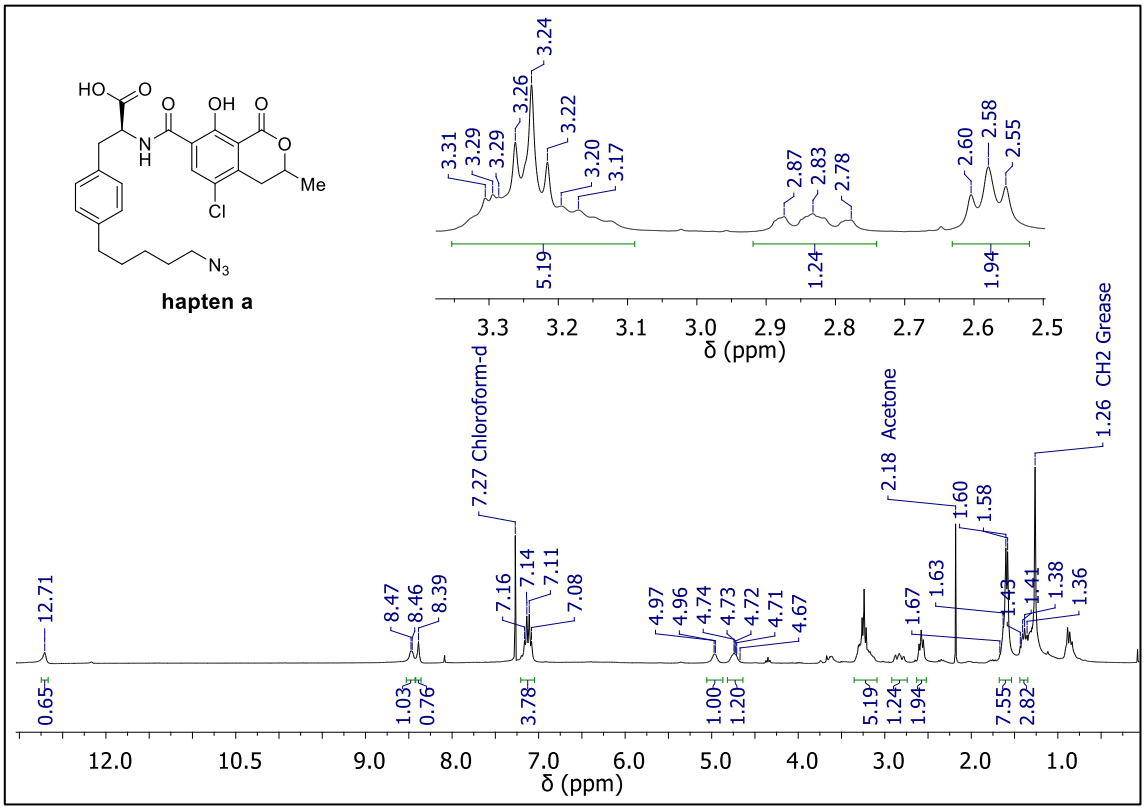
^{1}H-NMR spectrum of intermediate **11**.



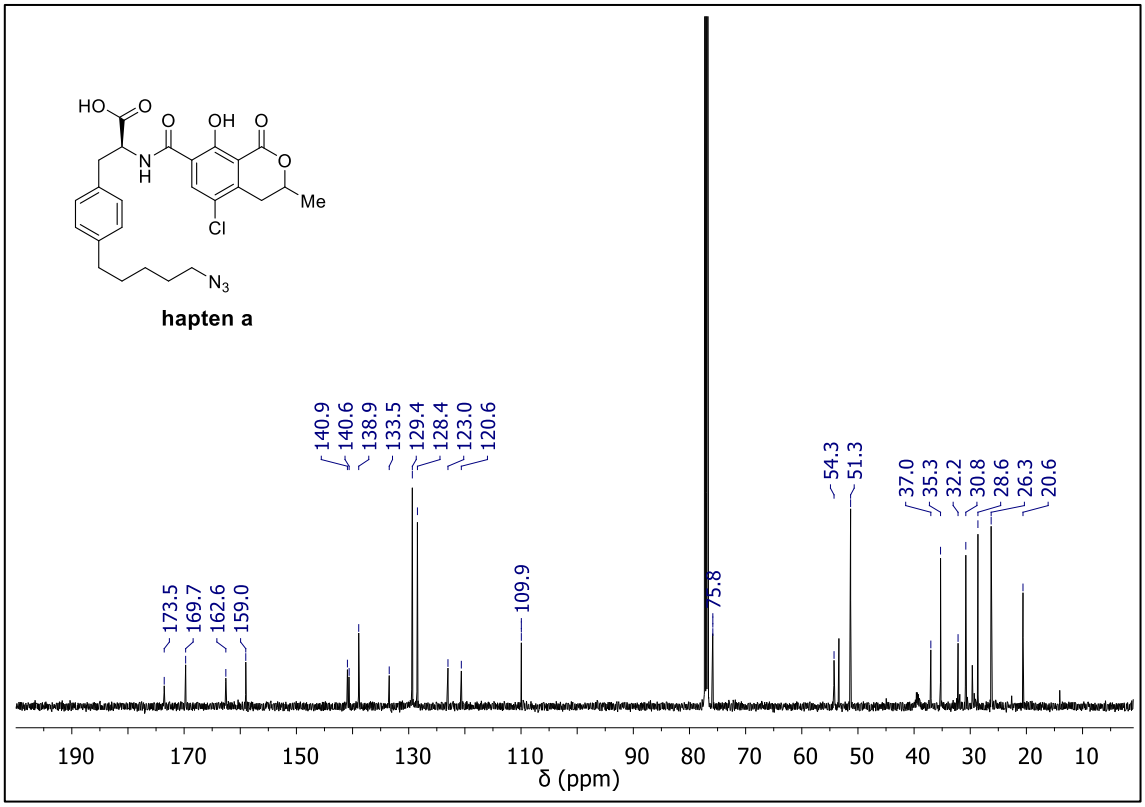
^{13}C-NMR spectrum of intermediate **11**.



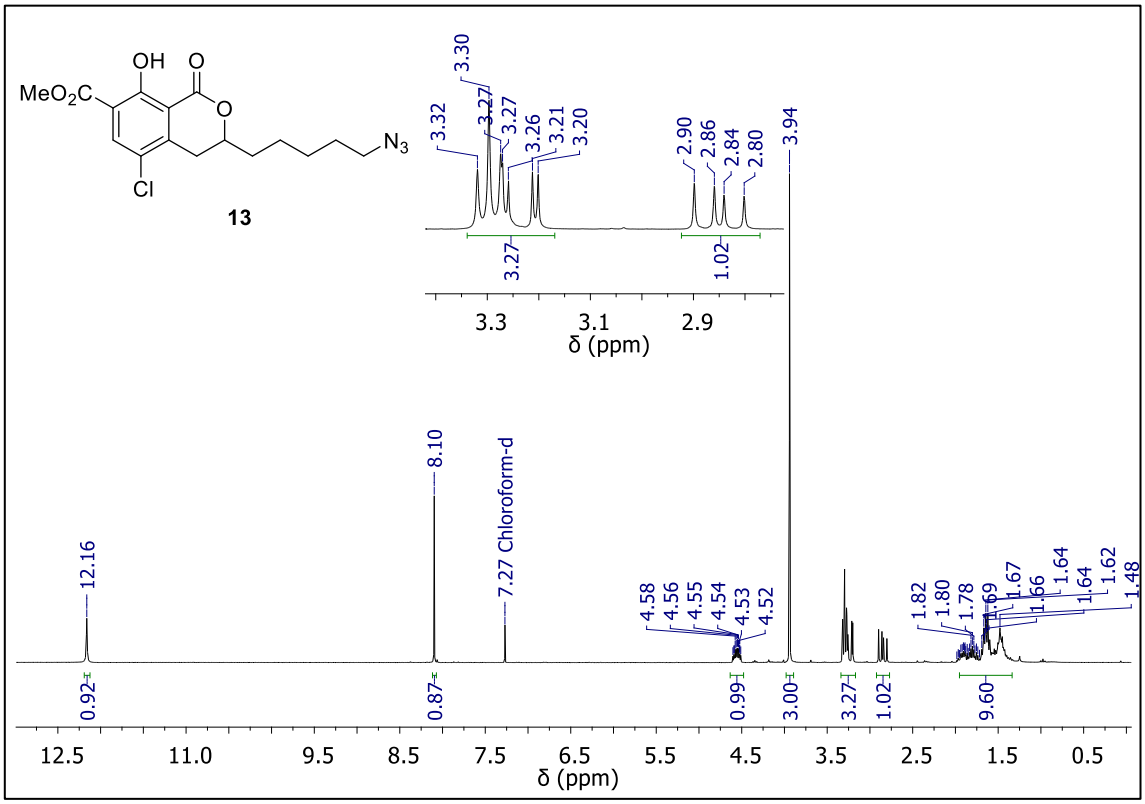
^{1}H-NMR spectrum of intermediate **hapten a**.



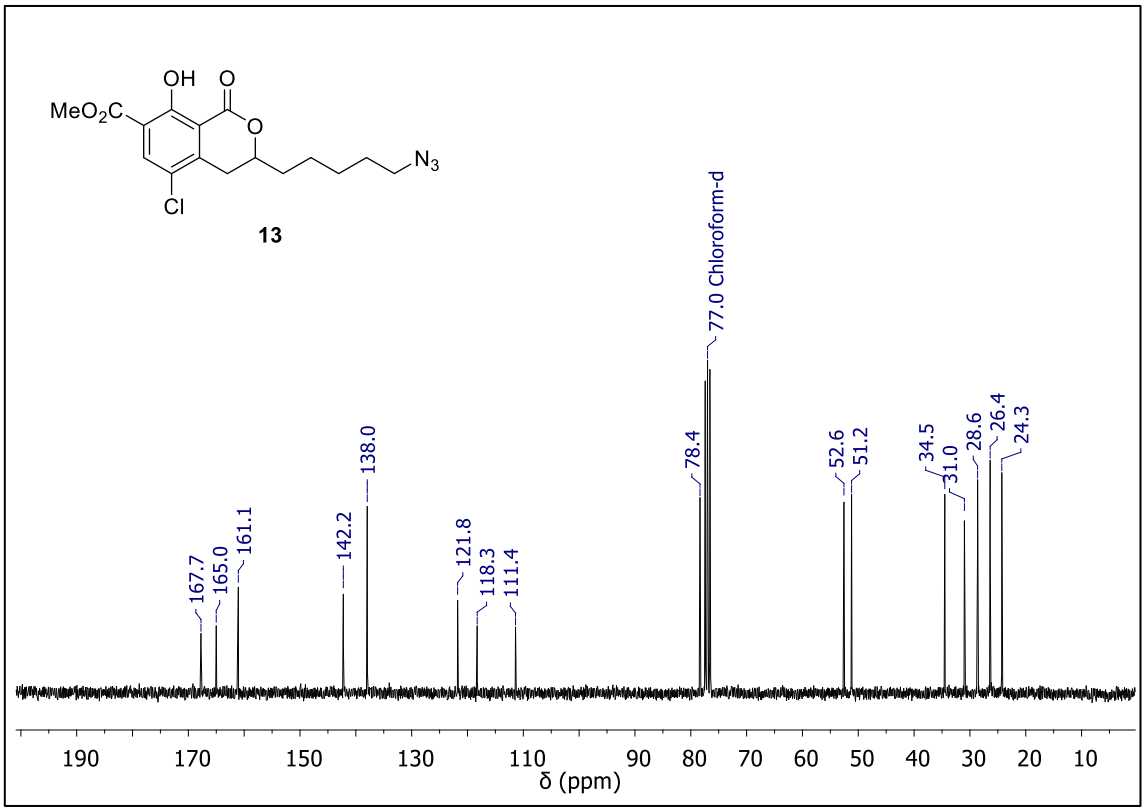
^{13}C-NMR spectrum of intermediate **hapten a**.



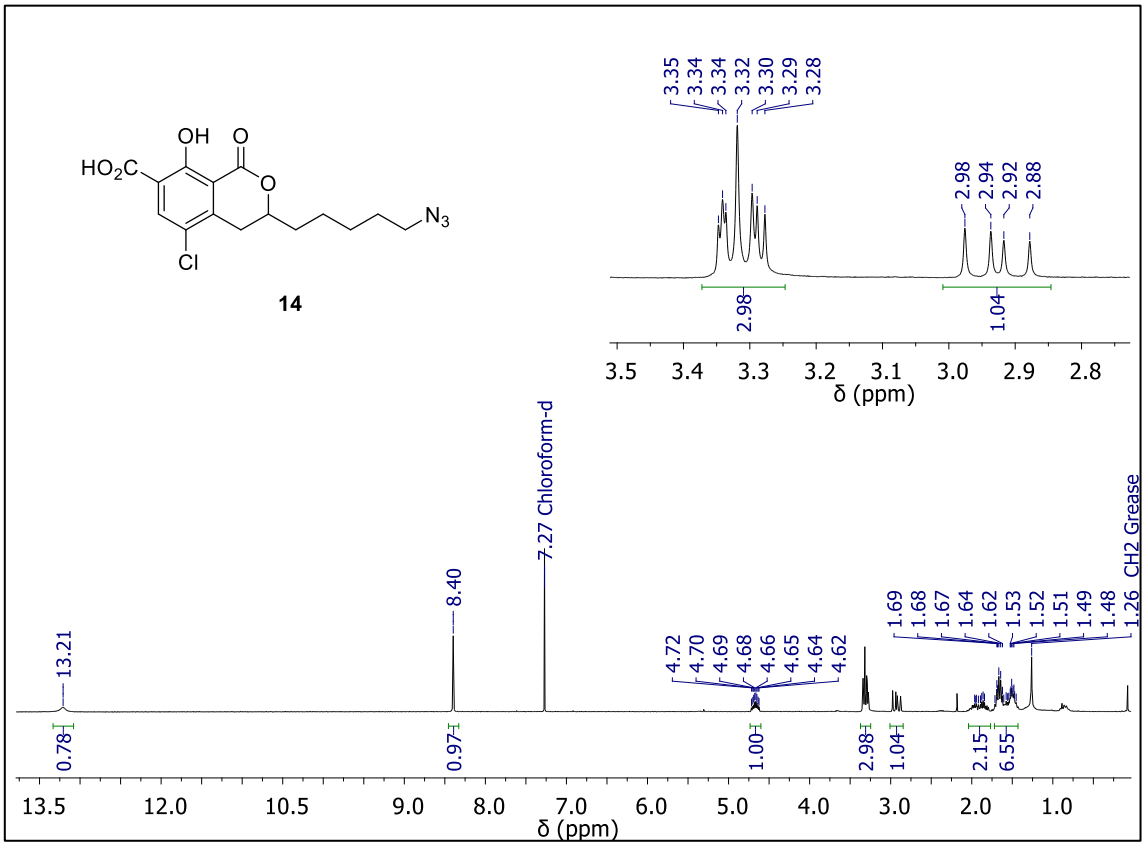
^{1}H-NMR spectrum of intermediate **13**.



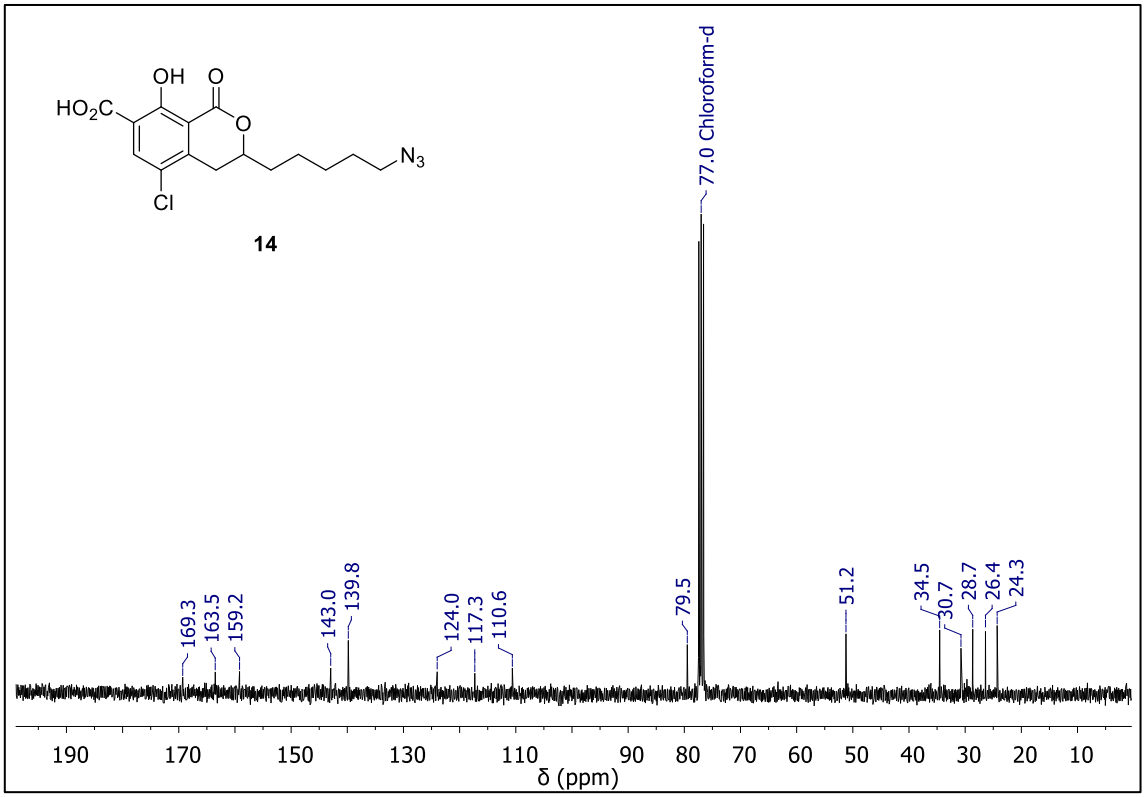
^{13}C-NMR spectrum of intermediate **13**.



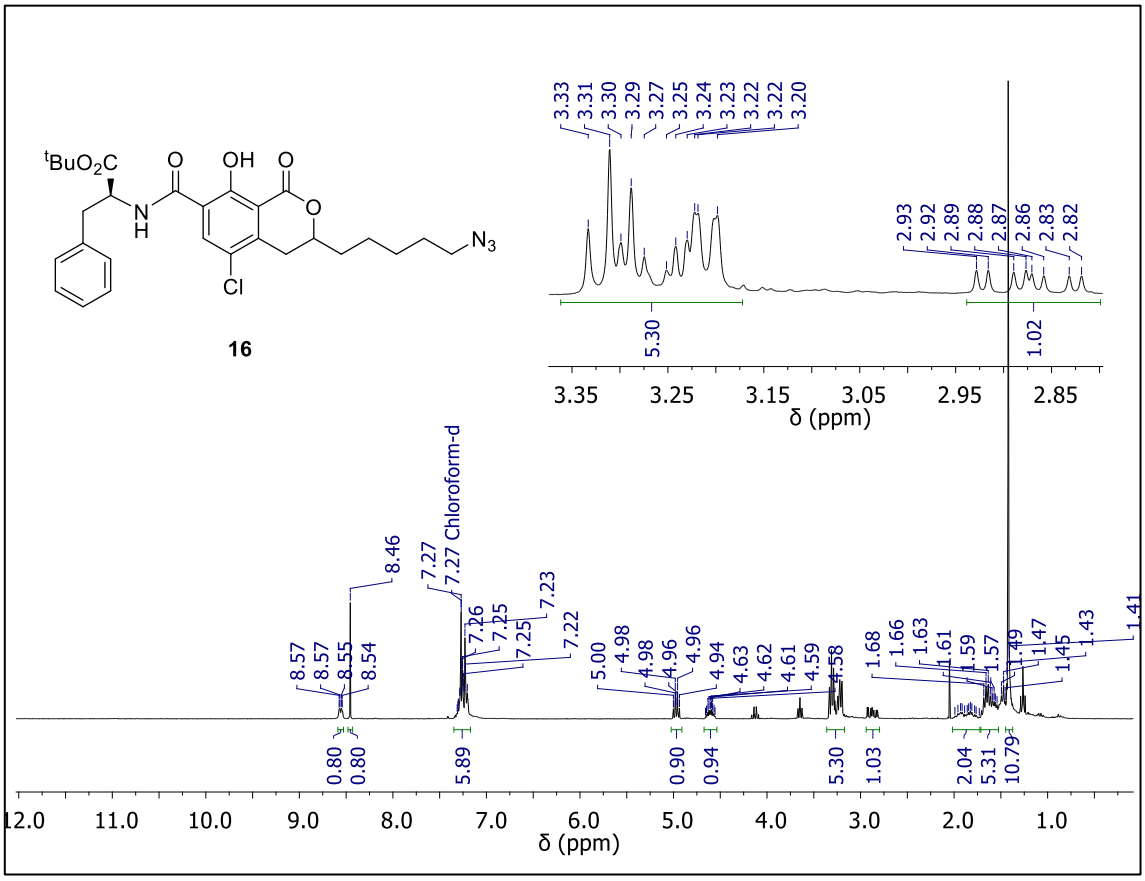
^{1}H-NMR spectrum of intermediate **14**.



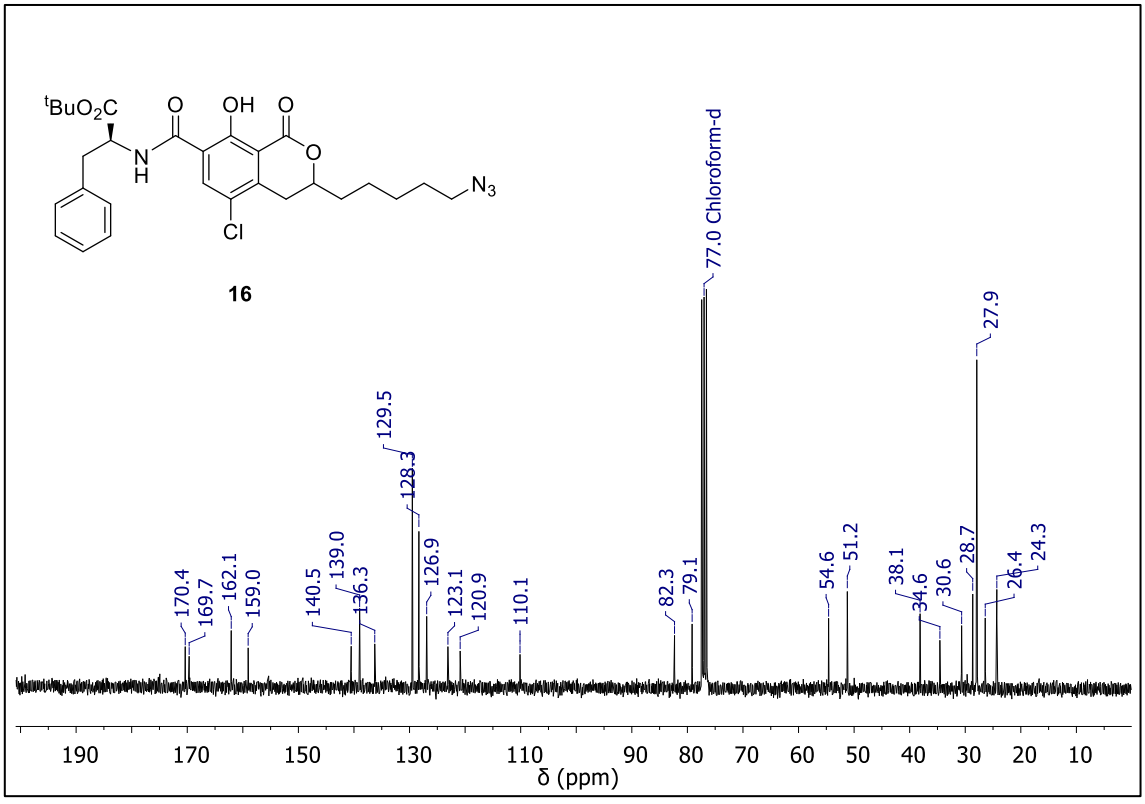
^{13}C-NMR spectrum of intermediate **14**.



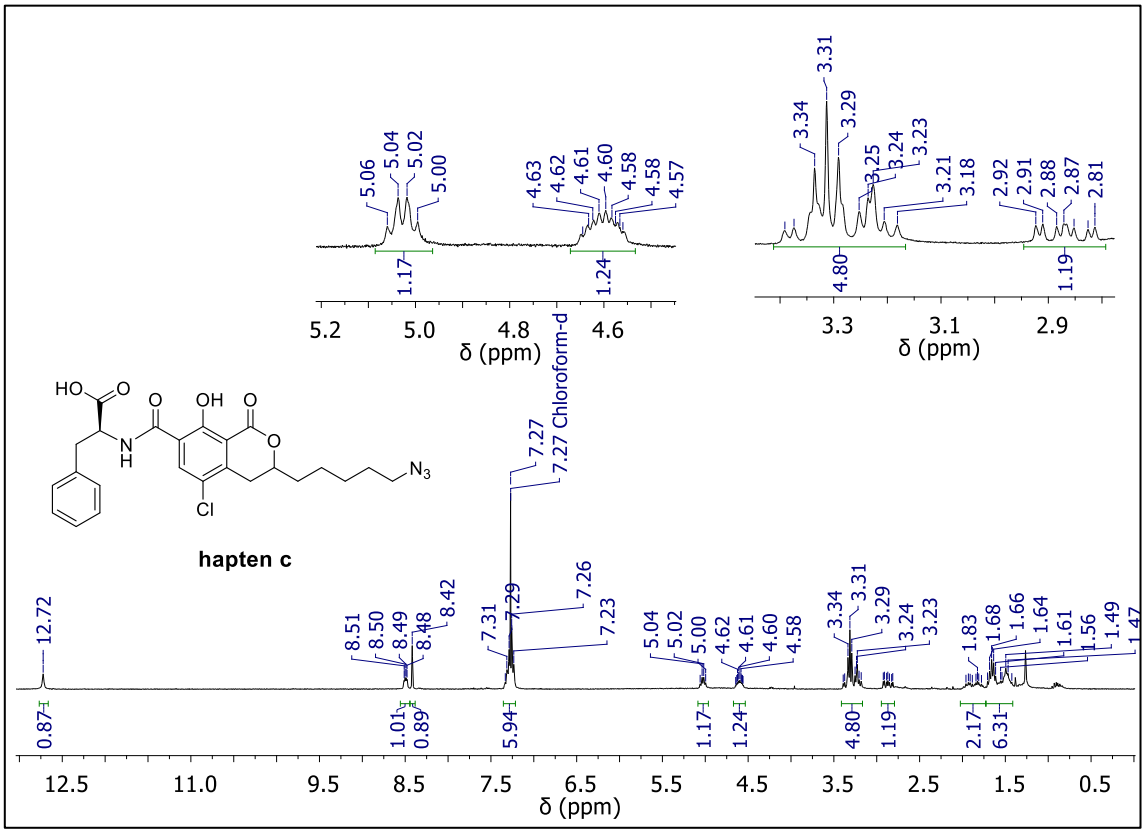
^{1}H-NMR spectrum of intermediate **16**.



^{13}C-NMR spectrum of intermediate **16**.



^{1}H-NMR spectrum of intermediate **hapten c**.



^{13}C-NMR spectrum of intermediate **hapten c**.