

ELECTRONIC SUPPLEMENTARY INFORMATION FOR:

Covalent attachment of antagonists to the $\alpha 7$ nicotinic acetylcholine receptor: synthesis and reactivity of substituted maleimides

Joseph I. Ambrus,^a Jill I. Halliday,^a Nicholas Kanizaj,^a Nathan Absalom,^b Kasper Harpsøe,^c Thomas Balle,^d Mary Chebib^b and Malcolm D. McLeod^a

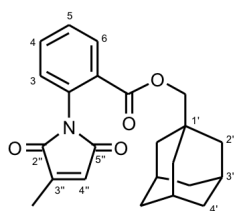
^a Research School of Chemistry, Australian National University, Canberra, ACT 0200, Australia, ^b Faculty of Pharmacy, The University of Sydney, NSW 2006, Australia, ^c NNF Centre for Protein Research and ^d Department of Drug Design and Pharmacology, Faculty of Health and Medical Sciences, University of Copenhagen, Universitetsparken 2, 2100 Copenhagen, Denmark.

E-mail: malcolm.mcleod@anu.edu.au Fax: (+61) 2 6125 8114; Tel: (+61) 2 6125 3504

General Experimental

Infrared absorption (IR) spectra were obtained using a Perkin–Elmer Spectrum One FTIR spectrometer. Compounds were prepared as a thin film between 0.5 cm sodium chloride plates. Absorption maxima (ν_{\max}) are expressed in wavenumbers (cm^{-1}). ¹H Nuclear magnetic resonance spectra were recorded using a Varian Mercury 300 (300 MHz) or Varian Mercury 400 (400 MHz) spectrometer at 25 °C, and are recorded in parts per million (ppm) downfield shift from tetramethylsilane ($\delta_{\text{TMS}} = 0$), using residual chloroform solvent (δ 7.26), methanol (δ 3.31) or DMSO (δ 2.50) as internal reference. The data is reported as chemical shift (δ_{H}), multiplicity (s = singlet, br = broad, d = doublet, t = triplet, q = quartet, m = multiplet), coupling constant (J Hz), assignment, and relative integral. ¹³C Nuclear magnetic resonance spectra were recorded using a Varian Mercury 300 (75 MHz) or Varian Mercury 400 (100 MHz) spectrometer at 25 °C with complete proton decoupling. Data is expressed in parts per million (ppm) downfield relative to TMS ($\delta_{\text{TMS}} = 0$) using deuterated chloroform (δ 77.16) as an internal reference and is reported as chemical shift (δ_{C}). High and low resolution electron impact ionization (EI) mass spectra were recorded using a Micromass VG Autospec mass spectrometer. Low and high resolution electrospray ionisation (ESI) mass spectra were recorded using a Micromass ZMD LR mass spectrometer or a Waters LCT Premier XE mass spectrometer respectively. Analytical thin layer chromatography (TLC) was performed using 0.2 mm thick aluminium backed pre-coated silica gel plates (Merck Kieselgel 60 F254). Flash chromatography was carried out using Merck Kieselgel 60 (230–400 mesh ASTM), under a positive pressure of nitrogen. Solvent compositions were mixed v/v as specified. All solvents and reagents were purified according to standard literature procedures.

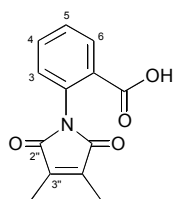
Synthesis of adamant-1-ylmethyl 2-(3-methyl-2,5-dihydro-2,5-dioxo-1H-pyrrol-1-yl)benzoate 7



A solution of 2-(3-methyl-2,5-dihydro-2,5-dioxo-1H-pyrrol-1-yl)benzoic acid¹ (250.3 mg, 1.1 mmol), adamant-1-ylmethanol (150.0 mg, 0.90 mmol), DCC (223.4 mg, 1.1 mmol) and DMAP (11.1 mg, 91 μ mol) in dichloromethane was stirred at room temperature for 1 h and heated at 35 °C for a further 1 h to aid consumption of the starting alcohol. The crude reaction mixture was filtered and the solvent removed *in vacuo*. The crude material was purified by flash column chromatography using 15% ethyl acetate/hexanes as eluant to afford the product **7** as a white solid (269.2 mg, 79%).

mp 141–142 °C; ν_{\max} (NaCl)/cm⁻¹ 2903, 2848, 1713, 1493, 1453, 1257, 1106, 1083, 984, 854, 757, 698, 616; δ_{H} (400 MHz, CDCl₃) 8.10 (dd, J = 7.6, 1.6 Hz, H-6, 1H), 7.63 (td, J = 7.6, 2.0 Hz, H-4, 1H), 7.51 (td, J = 7.6, 1.2 Hz, H-5, 1H), 7.29 (dd, J = 7.6, 1.2 Hz, H-3, 1H), 6.50 (q, J = 1.6 Hz, H-4', 1H), 3.81 (s, CH₂O, 2H), 2.16 (d, J = 2.0 Hz, CH₃, 3H), 1.99 (bs, H-3', 3H), 1.74–1.64 (m, H-4', 6H), 1.56 (d, J = 2.0 Hz, H-2', 6H); δ_{C} (100 MHz, CDCl₃) 170.9 (C2''), 169.9 (C5''), 165.0 (C=O), 146.3 (C3''), 133.2 (C4), 131.9 (C2), 131.5 (C6), 130.5 (C3), 129.1 (C5), 128.4 (C1), 128.0 (C4'), 79.4 (CH₂O), 39.4 (C2'), 37.0 (C4'), 33.4 (C1'), 28.1 (C3'), 11.3 (CH₃); **HRMS** (EI) found 379.1787, C₂₃H₂₅NO₄ (M⁺) requires 379.1784.

Synthesis of 2-(3,4-dimethyl-2,5-dihydro-2,5-dioxo-1H-pyrrol-1-yl)benzoic acid 13

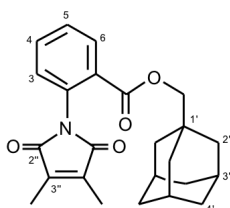


A mixture of anthranilic acid (1.00 g, 7.29 mmol) and the 2,3-dimethylmaleic anhydride (1.38 g, 10.94 mmol) was heated at 160 °C in a sealed tube for 4 h. The crude reaction mixture was cooled and dissolved in ethyl acetate (20 mL). The organic layer was washed with aqueous hydrochloric acid solution (20 mL, 1 M), water (20 mL) and brine (20 mL).

The organic layer was concentrated *in vacuo* and separated by flash column chromatography, using 1% dichloromethane/methanol as eluant, to furnish product **13** as a white solid (0.60 g, 34%).

mp decomposed; ν_{\max} (NaCl)/cm⁻¹ 3199, 1724, 1691, 1494, 1409, 1223, 1130, 1093, 1073; δ_{H} (300 MHz, CDCl₃): 8.14 (dd, J = 7.8, 1.2 Hz, H-6, 1H), 7.68 (td, J = 7.5, 1.8 Hz, H-4, 1H), 7.50 (td, J = 7.5, 0.9 Hz, H-5, 1H), 7.31 (dd, J = 7.5, 1.2 Hz, H-3, 1H), 2.06 (s, CH₃, 6H), (COOH not observed); δ_{C} (100 MHz, CDCl₃): 171.1 (C2'), 170.3 (COOH), 138.1 (C3'), 134.1 (C4), 132.31 (C2), 132.28 (C6), 130.4 (C3), 128.9 (C5), 127.3 (C1), 9.1 (CH₃); **HRMS** (–ESI): found 244.0609, C₁₃H₁₀NO₄ ([M–H][–]) requires 244.0610.

Synthesis of adamant-1-ylmethyl 2-(3,4-dimethyl-2,5-dihydro-2,5-dioxo-1H-pyrrol-1-yl)benzoate 9

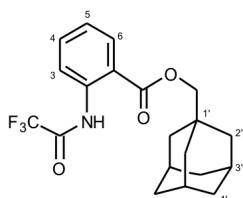


A solution of the carboxylic acid **13** (97.4 mg, 0.40 mmol), adamant-1-ylmethanol (55.0 mg, 0.33 mmol), DCC (81.9 mg, 0.40 mmol) and DMAP (4.0 mg, 33 μ mol) in dichloromethane was stirred at room temperature for 1 h and heated at 35 °C for a further 1 h to aid consumption of the starting alcohol. The crude reaction mixture was

filtered and the solvent removed *in vacuo*. The crude material was purified by flash column chromatography using 10% ethyl acetate/hexanes as eluant to afford the product **9** as a white solid (90.5 mg, 70%).

mp 120–121 °C; ν_{\max} (NaCl)/ cm^{-1} 2903, 2848, 1710, 1493, 1454, 1395, 1291, 1257, 1121, 1095, 984, 759, 729, 709; δ_{H} (400 MHz, CDCl_3) 8.09 (dd, $J = 8.0, 1.6$ Hz, H-6, 1H), 7.62 (td, $J = 8.0, 1.6$ Hz, H-4, 1H), 7.49 (td, $J = 8.0, 1.6$ Hz, H-5, 1H), 7.27 (dd, $J = 7.6, 1.2$ Hz, H-3, 1H), 3.79 (s, CH_2O , 2H), 2.05 (s, CH_3 , 6H), 1.98 (bs, H-3', 3H), 1.74–1.63 (m, H-4', 6H), 1.55 (d, $J = 2.4$ Hz, H-2', 6H); δ_{C} (100 MHz, CDCl_3) 171.2 (C2''), 165.2 (C=O), 138.0 (C3''), 133.4 (C4), 132.1 (C2), 131.5 (C6), 130.4 (C3), 128.8 (C5), 128.5 (C1), 74.9 (CH_2O), 39.4 (C2'), 37.0 (C4'), 33.4 (C1'), 28.1 (C3'), 9.0 (CH_3); **HRMS** (EI) found 393.1942, $\text{C}_{24}\text{H}_{27}\text{NO}_4$ (M^{+}) requires 393.1940.

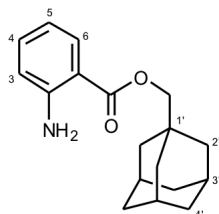
Synthesis of adamant-1-ylmethyl 2-(2,2,2-trifluoroacetamido)benzoate **14**



A solution of the *N*-(2,2,2-trifluoroacetyl)anthranilic acid **11** (841.4 mg, 3.61 mmol), adamant-1-ylmethanol (500.0 mg, 3.01 mmol), DCC (744.6 mg, 3.61 mmol) and DMAP (36.7 mg, 0.30 mmol) in dichloromethane was stirred for 3 d at 30 °C. The crude reaction mixture was filtered and concentrated *in vacuo*. The crude material was separated by flash column chromatography using 5% ethyl acetate/hexane as eluant to afford the title compound **14** as a white solid (1.15 g, 100%).

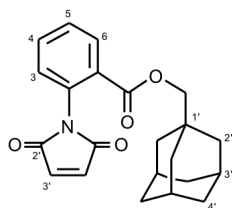
mp 107.5–108.0 °C; ν_{\max} (NaCl)/ cm^{-1} 2910, 2849, 1729, 1686, 1537, 1307, 1285, 1262, 1187, 1164, 759; δ_{H} (400 MHz, CDCl_3) 12.39 (bs, NH, 1H), 8.66 (dd, $J = 8.8, 1.2$ Hz, H-3, 1H), 8.12 (dd, $J = 8.0, 1.6$ Hz, H-6, 1H), 7.63 (td, $J = 7.6, 1.6$ Hz, H-4, 1H), 7.26 (td, $J = 7.6, 1.6$ Hz, H-5, 1H), 3.97 (s, CH_2O , 2H), 2.04 (bs, H-3', 3H), 1.79–1.68 (m, H-4', 6H), 1.64 (d, $J = 2.8$ Hz, H-2', 6H); δ_{C} (100 MHz, CDCl_3) 168.4 (C=O), 155.3 (CF_3CO), 139.2 (C2), 135.0 (C4), 131.0 (C6), 124.8 (C5), 120.8 (C3), 116.7 (C1), 115.9 (q, $J_{\text{C-F}} = 287.2$ Hz, CF_3), 75.4 (CH_2O), 39.5 (C2'), 37.0 (C4'), 33.6 (C1'), 28.1 (C3'); **HRMS** (EI) found 381.1553, $\text{C}_{20}\text{H}_{22}\text{F}_3\text{NO}_3$ (M^{+}) requires 381.1552.

Synthesis of adamant-1-ylmethyl 2-aminobenzoate **12**



To a solution of sodium borohydride (108 mg, 2.85 mmol) in ethanol (10 mL) was added trifluoroacetamide **14** (1.09 g, 2.85 mmol) in ethanol (5 mL). The reaction was stirred at room temperature for 1 h. The reaction was quenched by addition of water (15 mL) at 0 °C. The aqueous layer was extracted with dichloromethane (3 × 10 mL) and then concentrated *in vacuo* to remove any remaining ethanol. The aqueous layer was further extracted with dichloromethane (3 × 5 mL) and the organic layers were combined, dried (MgSO_4) and concentrated *in vacuo* to give a white solid. The crude solid was separated by flash column chromatography using 2.5% ethyl acetate/hexane as eluant to afford the desired product **12** as a white solid (634.3 mg, 78%) which was used directly in preparing the maleimides below.

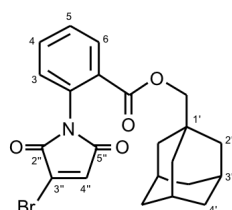
Synthesis of adamant-1-ylmethyl 2-(2,5-dihydro-2,5-dioxo-1H-pyrrol-1-yl)benzoate 5



To a solution of the maleic anhydride (230.2 mg, 2.35 mmol) in diethyl ether (10 mL) was added the amine **12** (670.0 mg, 2.35 mmol). The solution was left to stir at room temperature for 30 minutes at which point the solvent was concentrated *in vacuo*. The intermediate amide was added to a solution of sodium acetate (77.0 mg, 0.94 mmol) in acetic anhydride (10 mL). The reaction was stirred at 85 °C for 30 min, at which point it was poured onto a suspension of ice in saturated aqueous sodium bicarbonate solution (15 mL). The mixture was left to neutralise for 1 h, and the aqueous layer was extracted with dichloromethane (3 × 15 mL). The organic layers were combined, dried (MgSO₄) and concentrated *in vacuo* to give a crude yellow solid. The crude material was separated using flash column chromatography using 10% ethyl acetate/hexane as eluant, to afford the title compound **5** as a pale white solid (175.1 mg, 20%).

mp 144–145 °C; ν_{\max} (NaCl)/cm⁻¹ 2903, 2848, 1717, 1493, 1454, 1392, 1258, 1153, 1137, 1088, 827, 707, 688; δ_{H} (400 MHz, CDCl₃) 8.13 (d, J = 8.0, 1.6 Hz, H-6, 1H), 7.66 (td, J = 7.6, 1.6 Hz, H-4, 1H), 7.53 (td, J = 7.6, 1.2 Hz, H-5, 1H), 7.31 (dd, J = 8.4, 1.2 Hz, H-3, 1H), 6.87 (s, H-3'', 2H), 3.82 (s, CH₂O, 2H), 2.00 (bs, H-3', 3H), 1.75–1.64 (m, H-4', 6H), 1.57 (d, J = 2.8 Hz, H-2', 6H); δ_{C} (100 MHz, CDCl₃) 170.0 (C2''), 164.9 (C=O), 134.7 (C3''), 133.4 (C4), 131.7 (C6), 131.6 (C2), 130.7 (C3), 129.3 (C5), 128.4 (C1), 74.9 (CH₂O), 39.5 (C2'), 37.0 (C4'), 33.5 (C1'), 28.1 (C3'); **HRMS** (EI) found 365.1649; C₂₂H₂₃NO₄ (M⁺) requires 365.1627.

Synthesis of adamant-1-ylmethyl 2-(3-bromo-2,5-dihydro-2,5-dioxo-1H-pyrrol-1-yl)benzoate 6

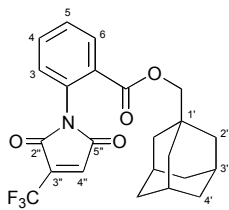


To a solution of bromomaleic anhydride (282.5 mg, 1.6 mmol) in diethyl ether (5 mL) was added the amine **12** (455.6 mg, 1.6 mmol). The solution was left to stir at room temperature for 30 minutes at which point the solvent was concentrated *in vacuo*. The intermediate amide was added to a solution of sodium acetate (52.4 mg, 0.64 mmol) in acetic anhydride (10 mL). The reaction was stirred at 85 °C for 30 min, at which point it was poured onto a suspension of ice in saturated aqueous sodium bicarbonate solution (15 mL). The mixture was left to neutralise for 1 h, and the aqueous layer was extracted with dichloromethane (3 × 15 mL). The organic layers were combined, dried (MgSO₄) and concentrated *in vacuo* to give a crude yellow solid. The crude material was separated using flash column chromatography using 10% ethyl acetate/hexane as eluant, to afford the title compound **6** as a pale white solid (429.0 mg, 60%)

mp 164–166 °C; ν_{\max} (NaCl)/cm⁻¹ 2903, 2848, 1725, 1589, 1492, 1454, 1389, 1258, 1195, 1137, 1086, 754; δ_{H} (400 MHz, CDCl₃) 8.15 (dd, J = 7.6, 1.6 Hz, H-6, 1H), 7.67 (td, J = 7.6, 1.5 Hz, H-4, 1H), 7.56 (td, J = 8.0, 1.3 Hz, H-5, 1H), 7.32 (dd, J = 7.6, 1.2 Hz, H-3, 1H), 7.05 (s, H-4'', 1H), 3.82 (s, CH₂O, 2H), 2.00 (bs, H-3', 3H), 1.76–1.64 (m, H-4', 6H), 1.57 (d, J = 2.8 Hz, H-2', 6H); δ_{C} (100 MHz, CDCl₃) 167.8 (C2''), 164.7 (C=O), 164.6 (C5''), 133.4 (C4), 132.5 (C4''), 132.1 (C3''), 131.7 (C6), 131.3 (C2), 130.7 (C3), 129.7

(C5), 128.2 (C1), 75.0 (CH₂O), 39.4 (C2'), 37.0 (C4'), 33.5 (C1'), 28.1 (C3'); **HRMS** (EI) found 443.0728, C₂₂H₂₂⁷⁹BrNO₄ (M⁺) requires 443.0732.

Synthesis of adamant-1-ylmethyl 2-(3-trifluoromethyl-2,5-dihydro-2,5-dioxo-1H-pyrrol-1-yl)benzoate **8**

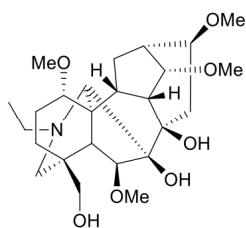


To a solution of the trifluoromethylmaleic anhydride (87.3 mg, 0.53 mmol) in dichloromethane (5 mL) was added the amine **12** (100.0 mg, 0.35 mmol). The solution was left to stir at room temperature for 30 minutes at which point the solvent was concentrated *in vacuo*. The intermediate amide was added to a solution of oxalyl chloride (222.1 mg, 1.7 mmol) in dichloromethane (25 mL) and dimethylformamide

(1 drop). The reaction was stirred at room temperature for 30 min, at which point it was concentrated *in vacuo* to give a black oil. The crude material was separated using flash column chromatography using 10% ethyl acetate/hexane as eluant, to afford the title compound **8** as an oil (7.6 mg, 5%).

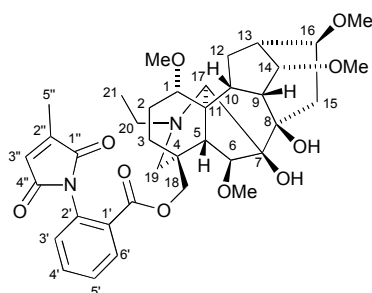
ν_{\max} (NaCl)/cm⁻¹ 2906, 2849, 1732, 1351, 1260, 1238, 1175; δ_{H} (800 MHz, CDCl₃) 8.17 (dd, $J = 8.0, 1.2$ Hz, H-6, 1H), 7.69 (td, $J = 7.6, 1.5$ Hz, H-4, 1H), 7.59 (td, $J = 7.4, 0.8$ Hz, H-5, 1H), 7.33 (dd, $J = 7.6, 0.8$ Hz, H-3, 1H), 7.17 (q, $J = 1.6$ Hz H-4'', 1H), 3.82 (s, CH₂O, 2H), 2.00 (br s, H-3', 3H), 1.76–1.65 (m, H-4', 6H), 1.57 (d, $J = 2.4$ Hz, H-2', 6H). δ_{C} (200 MHz, CDCl₃) 166.4, 164.5, 164.1, 135.6 (q, $^2J_{\text{CF}} = 37.5$ Hz, C3'), 133.7 (C4''), 133.5 (C4), 131.7 (C6), 130.64 (C2), 130.59 (C3), 129.9 (C5), 128.0 (C1), 119.3 (q, $^1J_{\text{CF}} = 271.6$ Hz, C5'), 75.0 (CH₂O), 39.3 (C2'), 36.9 (C4'), 33.4 (C1'), 28.0 (C3'); **HRMS** (EI) found 433.1506, C₂₃H₂₂F₃NO₄ (M⁺) requires 433.1501.

Synthesis of lycocotinine **15**^{3,4}



A solution of methyllycaconitine **1** (48.8 mg, 0.071 mmol) and ethanolic potassium hydroxide solution (2 mL, 2 M, 4.0 mmol) was stirred at room temperature for 3 h. The crude material was diluted with water (10 mL) and extracted with toluene (3 × 10 mL). The organic layer was separated, dried (MgSO₄) and concentrated to give lycocotinine **15** as a white solid (19.0 mg, 57%). All spectroscopic data matched that previously reported in the literature.^{3,4}

Synthesis of MLA maleimide **3**⁵

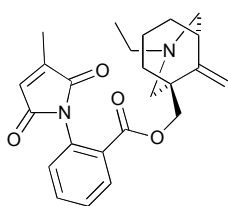


A solution of the 2-(3-methyl-2,5-dihydro-2,5-dioxo-1H-pyrrol-1-yl)benzoic acid¹ (10.3 mg, 0.044 mmol), lycocotinine **15** (19.0 mg, 0.041 mmol), EDC hydrochloride (9.4 mg, 0.049 mmol), and DMAP (0.1 mg, 0.8 nmol) in dichloromethane (2 mL) was stirred at room temperature for 16 h. The reaction was washed with water (5 mL) and the organic layer was dried (MgSO₄) and concentrated to give a crude yellow solid. The crude material was separated by flash column chromatography using 7% methanol/dichloromethane as eluent to yield the desired product **3** as a white solid (6.6 mg, 24%).

mp 124–126 °C; $[\alpha]_D^{20}$ +46.8 (*c* 0.59, CHCl₃); ν_{\max} (NaCl)/cm⁻¹ 3468, 2930, 2821, 1714, 1494, 1455, 1394, 1294, 1259, 1210, 1088; δ_H (800 MHz, CDCl₃) 8.02 (d, 1H, *J* = 7.8 Hz, H-6'), 7.67 (t, 1H, *J* = 7.7 Hz, H-4'), 7.53 (t, 1H, *J* = 7.5 Hz, H-5'), 7.32 (d, 1H, *J* = 7.8 Hz, H-3'), 6.50 (s, 1H, H-3''), 4.13 (d, 1H, *J* = 11.2 Hz, H-18 α), 4.02 (d, 1H, *J* = 11.2 Hz, H-18 β), 3.86 (s, 1H, H-6), 3.59 (t, 1H, *J* = 4.6 Hz, H-14), 3.41 (s, 3H, C14-OCH₃), 3.35 (s, 3H, C6-OCH₃), 3.32 (s, 3H, C16-OCH₃), 3.25 (s, 3H, C1-OCH₃), 3.21 (t, 1H, *J* = 7.9 Hz, H-16), 3.06 (t, 1H, *J* = 8.2 Hz, H-9), 2.97 (dd, 1H, *J* = 7.2, 9.8 Hz, H-1), 2.94–2.90 (m, 2H, H-17, H-20 α), 2.79 (m, 1H, H-20 β), 2.68 (d, 1H, *J* = 11.9 Hz, H-19 α), 2.60 (dd, 1H, *J* = 8.9, 15.2 Hz, H-15 α), 2.46 (dd, 1H, *J* = 5.2, 14.8 Hz, H-12 α), 2.40 (d, 1H, *J* = 11.6 Hz, H-19 β), 2.33 (dd, 1H, *J* = 4.9, 7.1 Hz, H-13), 2.19–2.13 (m, 4H, H-2 α , H-5''), 2.07 (m, 1H, H-2 β), 1.93 (m, 1H, H-10), 1.83 (m, 1H, H-12 β), 1.73 (m, 1H, H-3 α), 1.70 (s, 1H, H-5), 1.67 (dd, 1H, *J* = 6.9, 15.2 Hz, H-15 β), 1.52 (m, 1H, H-3 β) 1.05 (t, 3H, *J* = 7.1 Hz, H-21) (OH not observed); δ_C (200 MHz, CDCl₃) 170.7 (C1''), 169.7 (C4''), 164.6 (Ar-C=O), 146.3 (C2''), 133.5 (C4'), 131.9 (C2'), 131.0 (C6'), 130.5 (C3'), 129.0 (C5'), 127.9 (C1'), 127.7 (C3''), 90.8 (C6), 88.5 (C7), 84.0 (C1), 83.9 (C14), 82.5 (C16), 77.5 (C8), 69.7 (C18), 64.5 (C17), 58.1 (C6-OCH₃), 57.8 (C14-OCH₃), 56.3 (C16-OCH₃), 55.8 (C1-OCH₃), 52.3 (C19), 51.0 (C20), 50.2 (C5), 49.0 (C11), 46.1 (C10), 43.2 (C9), 38.2 (C13), 37.5 (C4), 33.6 (C15), 32.1 (C3), 28.7 (C12), 26.1 (C2), 14.1 (C21), 11.2 (C5''); **HRMS** (+ESI): found 681.3386, C₃₇H₄₉N₂O₁₀ ([M+H]⁺) requires 681.3387.

The ¹H and ¹³C NMR assignments for the alkaloid region of compound **3** match the revised assignments reported for methyllycaconitine.⁶

Synthesis of ((1*S**,5*S**)-3-ethyl-9-methylidene-3-azabicyclo[3.3.1]nonan-1-yl)methyl 2-(3-methyl-2,5-dihydro-2,5-dioxo-1*H*-pyrrol-1-yl)benzoate **4**



A solution of 2-(3-methyl-2,5-dihydro-2,5-dioxo-1*H*-pyrrol-1-yl)benzoic acid¹ (913 mg, 4.0 mmol), ((1*S**,5*S**)-3-ethyl-9-methylidene-3-azabicyclo[3.3.1]nonan-1-yl)methanol^{7,8} (383 mg, 2.0 mmol), DCC (482 mg, 2.3 mmol) and DMAP (40.7 mg, 0.33 mmol) in acetonitrile (10 mL) was stirred at room temperature for 16 h. The crude reaction mixture was filtered and the solvent removed *in vacuo*. The residue was

redissolved in ethyl acetate (30 mL), washed with saturated aqueous sodium hydrogen carbonate solution, brine, dried (MgSO₄) and concentrated *in vacuo* to give the crude product. The crude material was purified by flash column chromatography using 20% ethyl acetate/hexane as eluant to afford the title compound **4** as a colourless oil (234 mg, 29%).

ν_{\max} (NaCl)/cm⁻¹ 2968, 2918, 2851, 2798, 1715, 1646, 1602; δ_H (300 MHz, CDCl₃) 8.08 (1H, m, H-6), 7.64 (1H, m, H-4), 7.50 (1H, m, H-5), 7.28 (1H, m, H-3), 6.51 (1H, m, H-4''), 4.73 (1H, s, C=CH_AH_B), 4.47 (1H, s, C=CH_AH_B), 4.16 (2H, s, OCH₂), 3.02–2.99 (2H, m, H-2'_A, H-4'_A), 2.77 (1H, m, H-7'_A), 2.41 (1H, m, H-5'), 2.32–2.16 (7H, m, H-2'_B, H-4'_B, C-3''-CH₃, NCH₂CH₃), 2.01–1.25 (5H, m, H-6', H-7'_B, H-8'), 1.04 (3H, t, *J* = 7.2 Hz, NCH₂CH₃); δ_C (75 MHz, CDCl₃) 170.9, 169.9, 164.9, 154.1, 146.4, 133.4, 131.9, 131.6, 130.5, 129.1, 128.1, 101.7, 71.0, 62.9, 60.5, 52.2, 41.8, 41.0, 36.5, 34.0, 21.5, 12.7, 11.4 (one carbon overlapping or obscured); **HRMS** (EI) found 408.2048, C₂₄H₂₈N₂O₄ (M⁺) requires 408.2049.

General procedure for the investigation of maleimide kinetics by ¹H NMR spectroscopy

A solution of the maleimide (25.0 μmol, 1 eq) in 0.5 ml of deuterated solvent was mixed with a solution of the thiol (250.0 μmol, 10 eq) in 0.5 mL of deuterated solvent at 25 °C. The ¹H NMR spectrum was immediately taken (0 min) and analysed for changes to the olefinic proton signal in the case of maleimides **5–8**, or de-symmetrisation of the dimethyl maleimide **9**. The reaction was followed for a 24 h period and the percentage of unreacted maleimide was determined by comparing the relative integration of the H6 aromatic proton for both the starting material/product to the H-4'' olefinic proton in the starting material only. The pseudo first order rate constant for the reaction of methyl maleimide with *N*-Ac-Cys-OH in d₆-DMSO at 22 °C was estimated by least squares curve fitting from the plot of ln([maleimide]/[maleimide]₀) against time as $k_{\text{obs}} 5.1 \pm 0.2 \times 10^{-3} \text{ s}^{-1}$.

Maleimides **5–9** were reacted with *N*-Ac-Cys-OH in d₆-DMSO, but due to limited supply of maleimide **8** only maleimides **5–7** and **9** were reacted with *N*-Boc-Cys-OMe in CD₃OD.

Site-directed mutagenesis and expression of recombinant α7, nAChR subunits in *Xenopus* oocytes

Rat α7 was cDNAs subcloned in pBS SK (+). Mutations were introduced into the vectors by designing sense and antisense nucleotide primers and using the Quickchange site-directed mutagenesis kit (Stratagene, La Jolla, USA), as previously described.⁹ Plasmids containing the mutant DNA were determined by DNA sequencing. All wildtype and mutant α7 mRNAs were transcribed *in vitro* using T7 mMessage mMachine™ transcription kit, and polyadenylated using the poly-A-tailing kit.

Xenopus laevis surgery, oocyte extraction and injection

The experiments were performed with Animal Ethics approvals from The University of Sydney. Female *X. laevis* were anaesthetised with tricaine (850 mg/500 mL) and ovarian lobes were surgically removed. The lobes were cut into small pieces and were rinsed thoroughly with oocyte releasing buffer 2 (OR2; 82.5 mM NaCl, 2 mM KCl, 1 mM MgCl₂, 5 mM HEPES (hemi-Na)). The lobes were digested with collagenase A (2 mg/mL in OR2; Boehringer Mannheim, Germany) at room temperature. The oocytes were stored in Frog Ringer buffer or ND96 wash solution (96 mM NaCl, 2 mM KCl, 1 mM MgCl₂, 1.8 mM CaCl₂, 5 mM HEPES (hemisodium salt) supplemented with 2.5 mM sodium pyruvate and 0.5 mM theophylline) until ready for injection. Stage V–VI oocytes were selected and microinjected with 2 ng mRNA in 50.6 nL. After injection, the oocytes were maintained at 18 °C in the presence of ND96 wash solution augmented with 50 μg/mL kanamycin.

We thank the Department of Pharmacology, the University of Sydney, for managing and maintaining the *Xenopus laevis* colony.

Electrophysiological recording of recombinant receptors

Whole-cell currents were measured using a two-electrode voltage clamp with a Digidata 1200, Geneclamp 500B amplifier together with a Powerlab/200 (AD Instruments, Sydney, Australia) and Chart version 3.5 for PC as previously described. The recording microelectrodes were filled with 3 M KCl and had resistance between 0.2 and 1 M Ω . Three to 5 days post-injection, oocytes held at -60 mV were used for recording. While recording, oocytes were superfused with calcium-free external solution contained 115 mM NaCl, 2.5 mM KCl, 1.8 mM BaCl₂, 10 mM HEPES until a stable base current was reached. Increasing concentrations of ACh were applied until a maximum concentration was reached.

To ensure desensitisation did not influence our results, buffer was washed for 20 minutes between ACh applications, or compound and ACh applications to α 7 nAChRs, and 6 minutes for α 7 L9'T nAChRs unless otherwise stated in the text.

The rate of covalent modification of cysteine residues by the reactive MLA analogues was measured at 22 °C by application of an EC₅₀ concentration of ACh twice to the receptor prior to the application of the MLA reactive compound. The peak currents (I_{50}) were required to be within 10% of each other before the reactive compound was applied for 30 seconds. An EC₅₀ concentration of ACh was applied and this sequence repeated, until there was no longer a reduction in the peak currents to ACh (**Supplementary Figure 1A**). The change in response to an EC₅₀ concentration of ACh after cumulative time of reagent addition (t) was expressed relative to the response to an EC₅₀ concentration of ACh prior to reagent addition, at $t = 0$, according to the following equation:

$$\text{Relative } I_{50} = I_{50(t)}/I_{50(0)}$$

The data expressed in this way were fitted to a single exponential decay to obtain an estimate of the first order rate constant (k) and maximum current reduction (Relative I_{∞}) (**Supplementary Figure 1 B and C**).

$$\text{Relative } I_{50} = I_{\infty} + (1 - I_{\infty})e^{-kt}$$

These were compared to the wild-type using a Student's t-test (**Figure 1**). The concentration of MLA maleimide **3** applied for the rate of reaction was 10 nM, however 1 μ M was applied for at least 3 minutes to the L9'T and L9'T:S189C mutants to ensure there was no reaction. The concentration of analogue maleimide **4** applied for the rate of reaction was 200 nM, however 1 μ M was applied for at least 9 minutes to the L9'T and L9'T:S189C mutants to ensure there was no reaction.

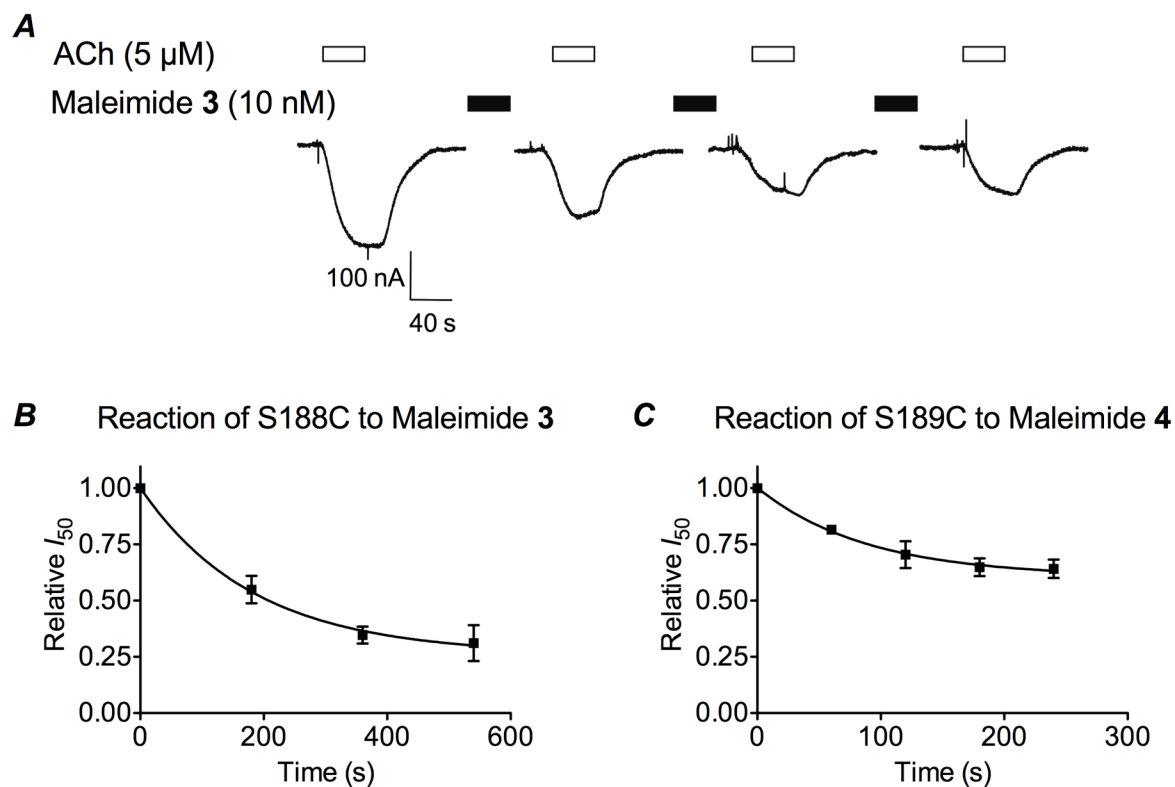
The N-terminal region of the α 7 nAChR contains only one unpaired cysteine residue C138. This residue resides within the β -sandwich core and is thus sterically hindered. The reaction of analogue maleimide **4** at this position is more likely compared to the more bulky MLA maleimide **3** which offers a potential explanation for the modest reduction in ACh elicited currents observed for the L9'T after treatment with analogue maleimide **4**.

Sequence alignment and rat $\alpha 7$ nAChR homology model

The sequence of the ligand-binding domain of rat $\alpha 7$ was retrieved from the Protein Knowledgebase¹⁰ (www.uniprot.org), entry Q05941. The structures and sequences of the templates were downloaded from the Protein Data Bank¹¹ (www.pdb.org): The mouse $\alpha 1$ nAChR ligand-binding domain with a toxin bound (PDB code: 2QC1),¹² AChBP mutated to resemble the human $\alpha 7$ nAChR with MLA bound (PDB code: 3SH1).¹³ Preparation of the templates was performed by manual deletion of unwanted protein chains, residues and heteroatom molecules. The alignment of the target and template sequences were initially created with T-COFFEE version 8.93¹⁴ and then manually edited according to the following considerations: Both templates were used in regions with structural similarity and otherwise 2QC1 was the template of choice except with respect to regions and residues that are conformationally different due to the bound antagonist or mutated residues (e.g. loop C). The alignment with specification of which templates were used in certain regions is depicted in **Supplementary Figure 2**. The dimeric rat $\alpha 7$ nAChR homology model was prepared with MODELLER version 9v10¹⁵ and MLA was included in the dimer interface from the 3SH1 template. We built 100 models and selected the best scoring model using the DOPE score¹⁶ included in MODELLER. The selected best scoring model is shown in **Figure 2** (cyan) after addition of hydrogens, optimisation of hydrogen bonds and a constrained geometry minimisation using the protein preparation tool in Maestro (Maestro, version 9.2, Schrödinger, LLC, New York, NY, 2011). The PDB file of MLA **1** bound to the $\alpha 7$ nAChR homology model is provided as ESI.

Sampling of Loop F

In order to make probable the reactivity of S188C:L9'T, Loop F of the selected model (M182-Y190) was sampled with a distance constraint between the S188 (sidechain oxygen) and the unsubstituted carbon in the succinimide moiety of MLA set to 3 Å. The loop was sampled using the extended loop sampling protocol in Prime (Prime, version 3.0, Schrödinger, LLC, New York, NY, 2011).^{17,18} The best scoring model (based on prime energy) was selected and it was checked that the sampled residues fell within the stereochemically allowed regions in a Ramachandran plot. An *in-silico* S188C mutation was introduced and the cysteine was connected to the succinimide moiety followed by constrained minimisation of the complex using the protein preparation tool in Maestro. The final loop model is shown in **Figure 2** (Blue). The PDB file of MLA maleimide **3** covalently attached to the loop sampled S188C $\alpha 7$ nAChR homology model is provided as ESI.



Supplementary Figure 1. *A* Raw current trace of $\alpha 7$ S188C:L9'T nAChR during the rate of reaction experiment. Acetylcholine (ACh) 5 μ M (white bars) was applied prior to and after addition of 10 nM MLA maleimide **3** for 180 s. *B* The peak current responses after MLA maleimide **3** addition were normalised to the response prior to reaction and plotted against time. An exponential curve was fitted to determine the rate constant, k , and maximum inhibition ($I_{(\infty)}$). *C* Plot and curve-fit of the reaction of 200 nM analogue maleimide **4** to $\alpha 7$ S189C:L9'T nAChR.

```
α7_rat (23) --GEFORRLYKELVKNYNPLERPVANDSQPLTVYFSLSLQIMDVDEKNO
3SH1 (1) HSQANLMRLKSDLFNRSPMYPGPTK--DDPLTVYLSFSLLDIVKADSSTN
2QC1 (1) --SEHETRLEAKLFEDYSSVVRPVEDHREIVQVTVGLQLIQLINVDEVNQ

α7_rat (71) VLTTNIWLQMSWTDHYLQWNMSEYPGVKNVRFDPGQIWKPDILLYNSADE
3SH1 (49) EVDLVYWEQQSWKLNLSLMDPNEYGNITDFRTSAADIWTPDITAYSSSTR-
2QC1 (49) IVTTNVRKQQWVDYNLKNPDDYGGVKKIHIPSEKIWRPDDVVLNNADG

α7_rat (121) RFDATFHNTNLVNASGHCQYLPPGIFKSSCYIDVRWFPPFDVQQCKLKFGS
3SH1 (98) PVQVLSPQNALVNSSGHVQYLP AQRLSFMCDPTGVD-SEEGATCAVKFGS
2QC1 (99) DFAIVKFTKVLDDYTGHIWTFPAIFKSYCEIIVTHFPFDEQNCMKLGT

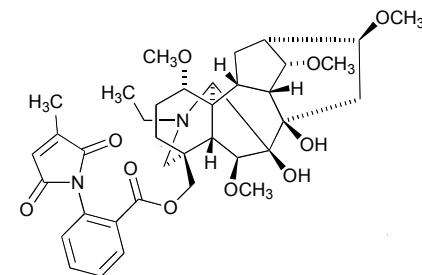
α7_rat (171) WSYGGWSLDLQ--MQEADISSYIPNGEWDLMGIPGKRNEKFYECC-KEYP
3SH1 (147) WSYGGWEIDLKTDTDQVDLSSY YASSKYEILSATQTRSERFYECC-KEYP
2QC1 (149) RTYDGSVAVAINPESDQPDLSNFMESGEWVIKEARGWKHWVVFYSCCPTTPY

α7_rat (218) PDVTYTVTMRRTT
3SH1 (196) PDVNLVVKFRERR
2QC1 (199) LDITYHFVMQRLP
```

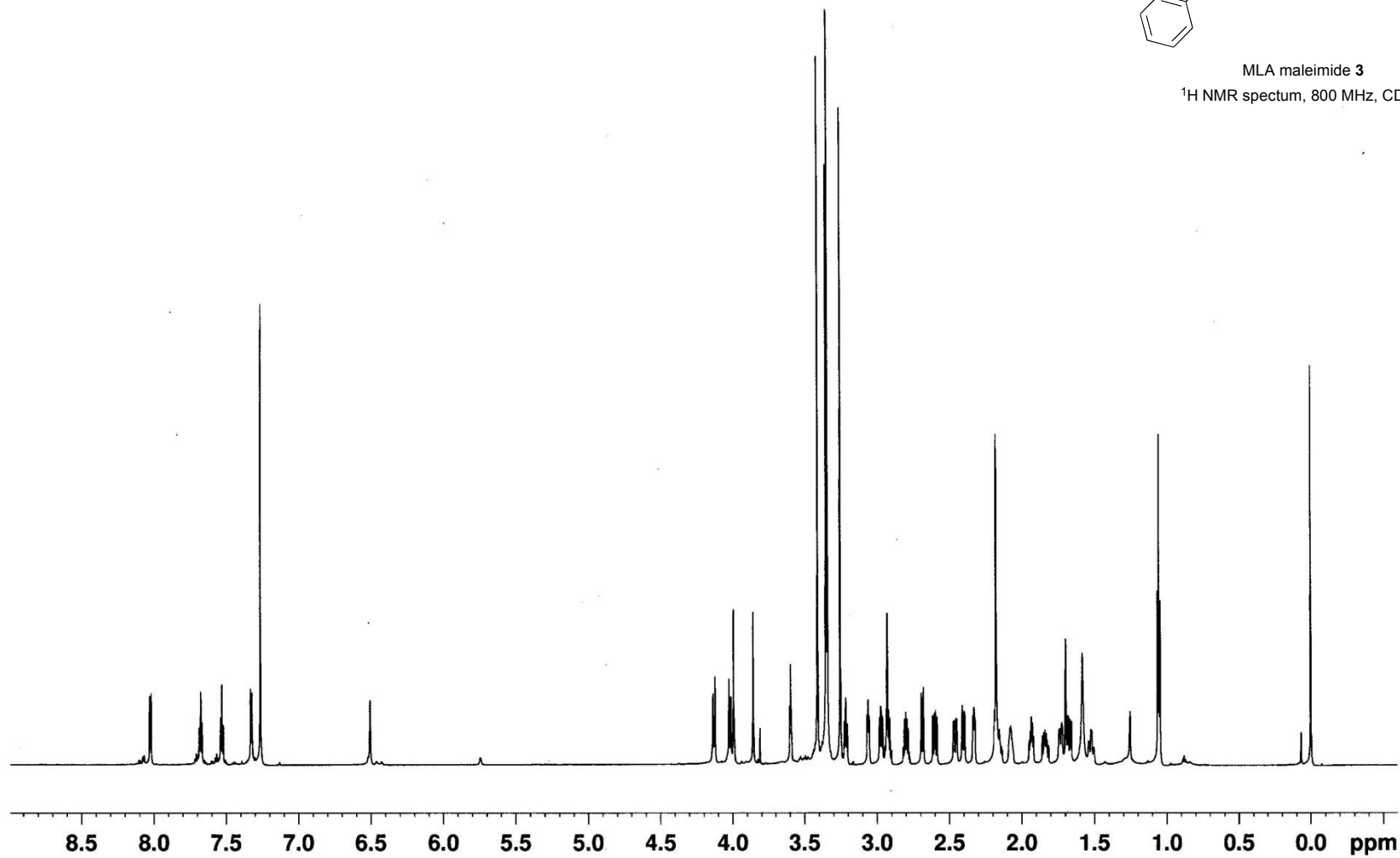
Supplementary Figure 2. Sequence alignment used for homology modelling. The sequence alignment of $\alpha 7$ to the two templates used to construct the dimeric $\alpha 7$ homology models. The residues shown in bold were used as templates while residues that were not modelled on a template or not used as template are shown in grey. Numbering follows the PDB files for 3SH1 (AChBP mutated to resemble human $\alpha 7$ nAChR with MLA bound) and 2QC1 (mouse $\alpha 1$ nAChR ligand binding domain with toxin bound) and is according to Q05941 at www.uniprot.org for rat $\alpha 7$.

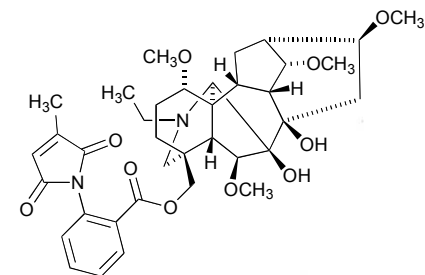
References

1. D. Barker, M. A. Brimble and M. D. McLeod, *Synthesis*, 2003, 656–658.
2. Z. Kozubková, M. Rouchal, M. Necas and R. Vicha, *Acta Crystallogr. Sect. E: Struct. Rep. Online*, 2010, **66**, 3262–3262.
3. A. H. Meriçli, S. Pırıldar, S. Süzgeç, L. Bitiş, F. Meriçli, H. Özçelik, J. Zapp and H. Becker, *Helv. Chim. Acta*, 2006, **89**, 210–217.
4. M. S. Yunusov, E. M. Tsyrlina, E. D. Khairitdinova, L. V. Spirikhin, A. Y. Kovalevsky and M. Y. Antipin, *Russ. Chem. Bull.*, 2000, **49**, 1629–1633.
5. K. R. Jennings, A. N. Starratt, P. Penaranda and B. G. Loughton, in *Progress in Neuropharmacology and Neurotoxicity of Pesticides and Drugs*, ed. D. J. Beadle, Royal Society of Chemistry, Cambridge, UK, 1999, pp. 163–174.
6. P. M. Shrestha and A. Katz, *J. Nat. Prod.*, 2004, **67**, 1574–1576.
7. D. Barker, M. D. McLeod, M. A. Brimble and G. P. Savage, *Tetrahedron Lett.*, 2001, **42**, 1785–1788.
8. D. Barker, M. A. Brimble and M. D. McLeod, *Tetrahedron*, 2004, **60**, 5953–5963.
9. G. X. J. Quek, D. Lin, J. I. Halliday, N. Absalom, J. I. Ambrus, A. J. Thompson, M. Lochner, S. C. R. Lummis, M. D. McLeod and M. Chebib, *ACS Chem. Neurosci.*, 2010, **1**, 796–809.
10. The UniProt Consortium, *Nucleic Acids Res.*, 2011, **39**, D214–D219.
11. H. M. Berman, J. Westbrook, Z. Feng, G. Gilliland, T. N. Bhat, H. Weissig, I. N. Shindyalov and P. E. Bourne, *Nucleic Acids Res.*, 2000, **28**, 235–242.
12. C. D. Dellisanti, Y. Yao, J. C. Stroud, Z.-Z. Wang and L. Chen, *Nat. Neurosci.*, 2007, **10**, 953–962.
13. Á. Nemezc and P. Taylor, *J. Biol. Chem.*, 2011, **286**, 42555–42565.
14. C. Notredame, D. G. Higgins and J. Heringa, *J. Mol. Biol.*, 2000, **302**, 205–217.
15. A. Šali and T. L. Blundell, *J. Mol. Biol.*, 1993, **234**, 779–815.
16. M.-y. Shen and A. Sali, *Protein Sci.*, 2006, **15**, 2507–2524.
17. M. P. Jacobson, D. L. Pincus, C. S. Rapp, T. J. F. Day, B. Honig, D. E. Shaw and R. A. Friesner, *Proteins Struct. Funct. Bioinf.*, 2004, **55**, 351–367.
18. M. P. Jacobson, R. A. Friesner, Z. Xiang and B. Honig, *J. Mol. Biol.*, 2002, **320**, 597–608.

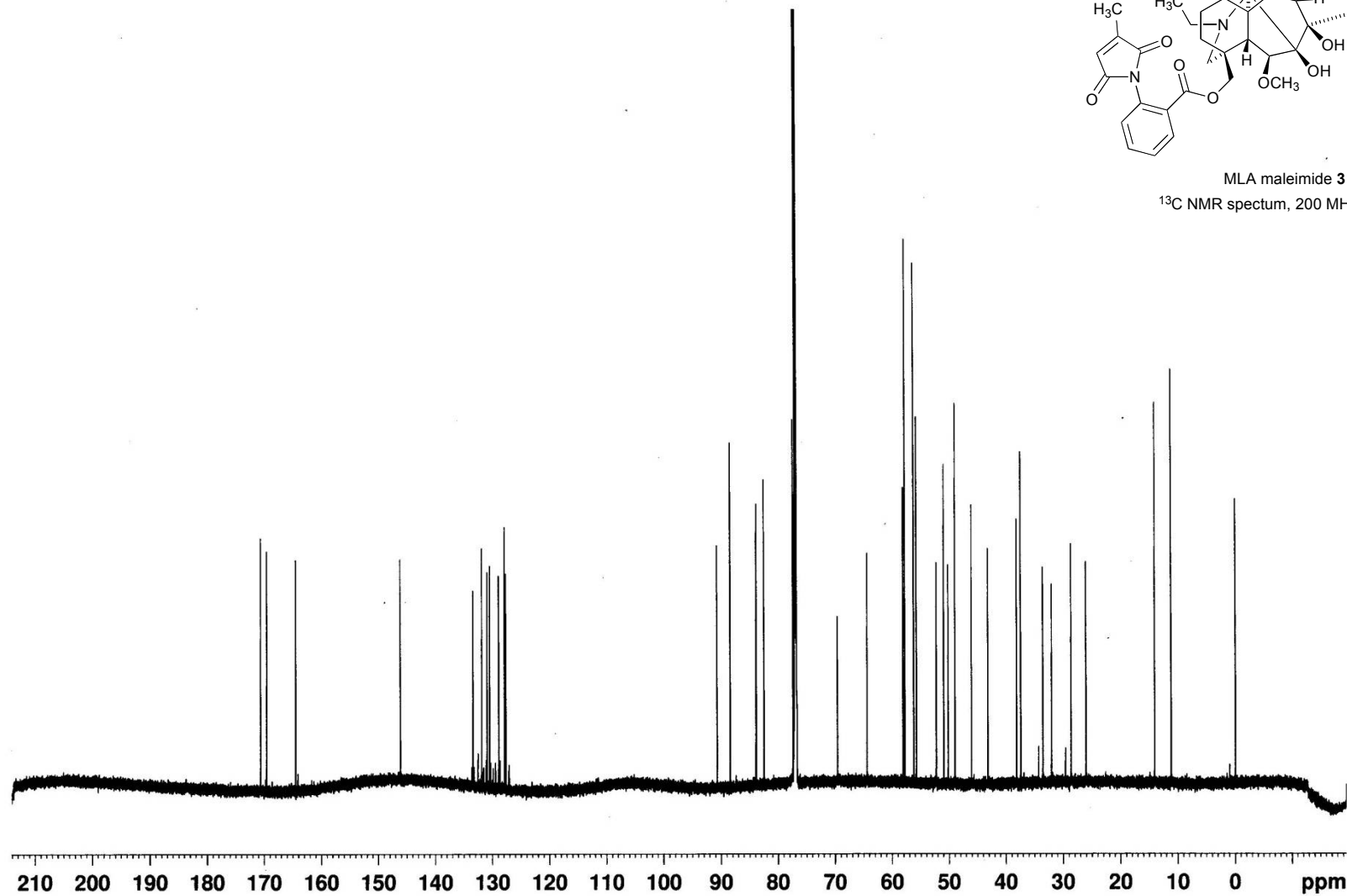


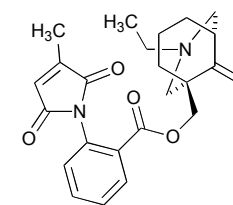
MLA maleimide 3
¹H NMR spectrum, 800 MHz, CDCl₃



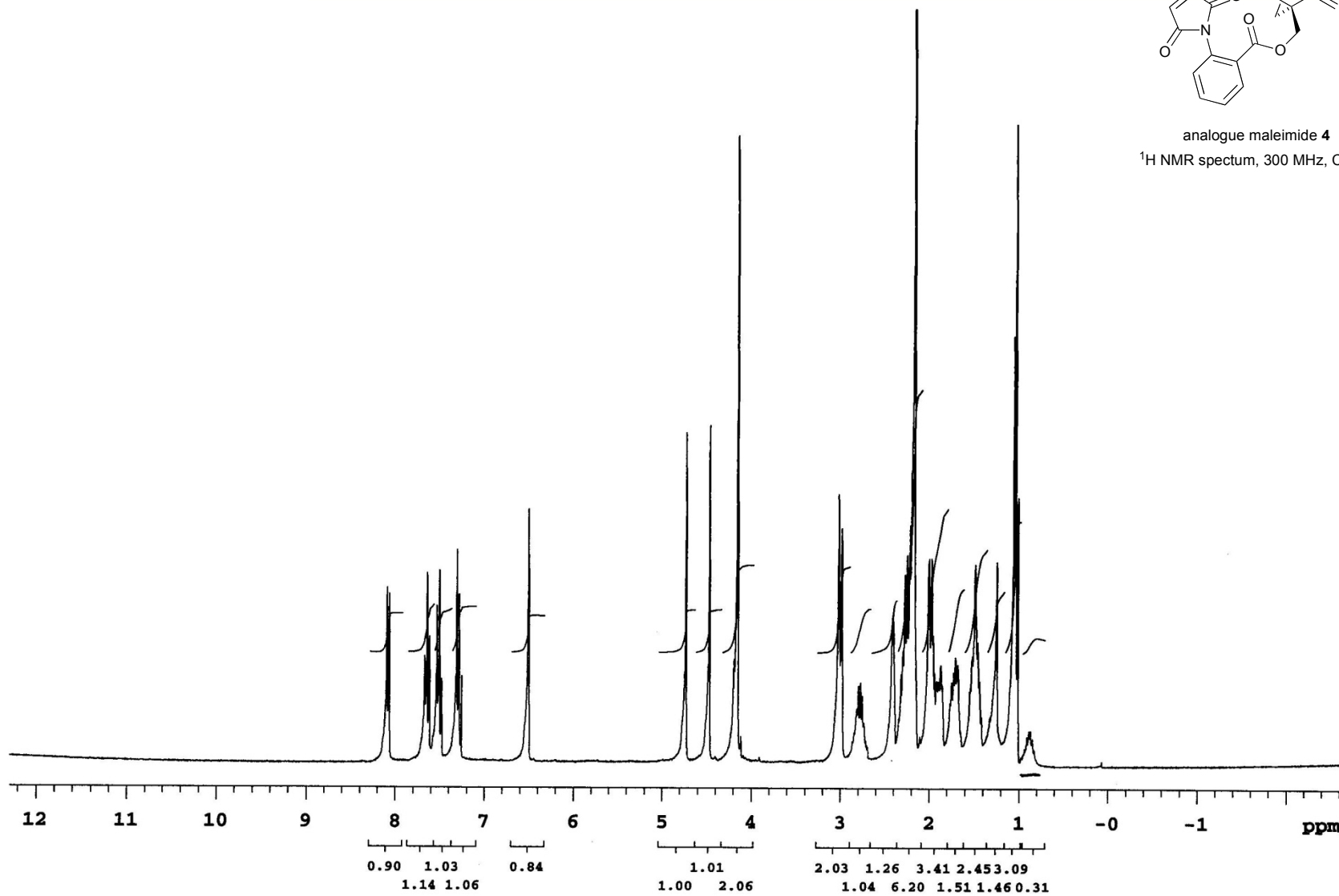


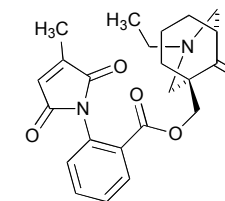
MLA maleimide 3
¹³C NMR spectrum, 200 MHz, CDCl₃





analogue maleimide **4**
¹H NMR spectrum, 300 MHz, CDCl₃





analogue maleimide 4
 ^{13}C NMR spectrum, 75 MHz, CDCl_3

