Supporting Information

A Gorge-Spanning, High-Affinity Cholinesterase Inhibitor to Explore β-Amyloid Plaques

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Synthesis of Compounds 3, 5, 8, and 10-12

Bis(2,4,6-trichlorophenyl) malonate (3). 2,4,6-Trichlorophenol (**1**; 80 mmol, 15.8 g) and malonic acid (**2**; 40 mmol, 4.16 g) were refluxed in phosphorus(V) oxychloride (107 mmol, 10 mL) for 3 h. After cooling to room temperature, the reaction mixture was quenched with ice water (50 mL), and the resulting precipitate was collected by suction filtration. The crude product was suspended in water (20 mL), saturated sodium hydrogen carbonate solution (5 mL) was added, and the suspension was stirred for 15 min. The undissolved material was filtered off and recrystallized from ethyl acetate to obtain **3** (15.2 g, 82%) as colorless cubes, mp 156 °C, lit.[1] 154-156 °C. ^{1}H NMR (DMSO-d_6) δ 4.04 (s, 2 H), 7.38 (s, 4 H); ^{13}C NMR (DMSO-d_6) δ 39.70, 128.74, 129.48, 132.69, 142.35, 161.44. EA found: C, 39.1; H, 1.5. $C_{15}H_6Cl_6O_4$ requires C, 39,1; H, 1.3%.

7-(Diethylamino)-4-hydroxy-2*H*-chromen-2-one (5). 3-Diethylaminophenol (**4**; 20.0 mmol, 3.30 g) and bis(2,4,6-trichlorophenyl) malonate (**3**; 20.0 mmol, 9.26 g) were dissolved in anhydrous toluene (50 mL) and heated to reflux for 2 h. Cooling to room temperature afforded a precipitate that was filtered off. The crude product was washed with toluene and hot petroleum ether to obtain **5** (3.53 g, 71%) as a light-brown powder, mp 241 °C, lit.[2] 238 °C. ^{1}H NMR (DMSO-d_6) δ 1.10 (app. s, 6 H), 3.39 (app. d, 4 H, J = 6.0 Hz), 5.24 (s, 1 H), 6.43 (s, 1 H), 6.64 (d, 1 H, J = 8.2 Hz), 7.53 (d, 1 H, J = 8.2 Hz), 11.86 (s, 1 H); ^{13}C NMR (DMSO-d_6) δ 12.42, 44.09, 86.27, 96.58, 103.64, 108.30, 124.24, 151.03, 156.27, 162.91, 166.62. EA found: C, 66.7; H, 6.95, N, 5.7. $C_{13}H_{15}NO_3$ requires C, 66.9; H, 6.5, N, 6.0%.

9-Chloro-1,2,3,4-tetrahydroacridine (8). Phosphorus(V) oxychloride (214 mmol, 20 mL) was slowly added to a mixture of anthranilic acid (**6**; 50 mmol, 6.87 g) and cyclohexanone (**7**; 50 mmol, 4.91 g) to be refluxed for 2 h. After cooling to room temperature, ice was added to the brown solution to quench excess phosphorus(V) oxychloride and the solution was neutralized using sodium hydroxide. The yellow suspension was diluted with water (250 mL) and extracted with ethyl acetate (3 × 250 mL). The combined organic phases were dried using anhydrous sodium

sulfate and subsequently evaporated *in vacuo*. Flash column chromatography using ethyl acetate and petroleum ether in a ratio of 1:2 provided **8** (7.08 g, 65%) from the first fractions, mp 67 °C, lit.[3] 67 °C. ^{1}H NMR (DMSO-d_6) δ 1.80-1.89 (m, 4 H), 2.90 (app. bs, 2 H), 2.99 (app. bs, 2 H), 7.72 (ddd, 1 H, *J* = 1.3, 7.0, 8.4 Hz), 7.61 (ddd, 1 H, *J* = 1.3, 7.0, 8.4 Hz), 7.91 (dd, 1 H, *J* = 0.7, 8.5 Hz), 8.07 (dd, 1 H, *J* = 1.0, 8.5 Hz); ^{13}C NMR (DMSO-d_6) δ 22.10, 22.12, 27.07, 33.66, 123.23, 124.62, 127.03, 128.66, 128.75, 129.59, 140.02, 146.28, 159.48. EA found: C, 71.8; H, 5.7, N, 6.5. $C_{13}H_{12}ClN$ requires C, 71.7; H, 5.6, N, 6.4%.

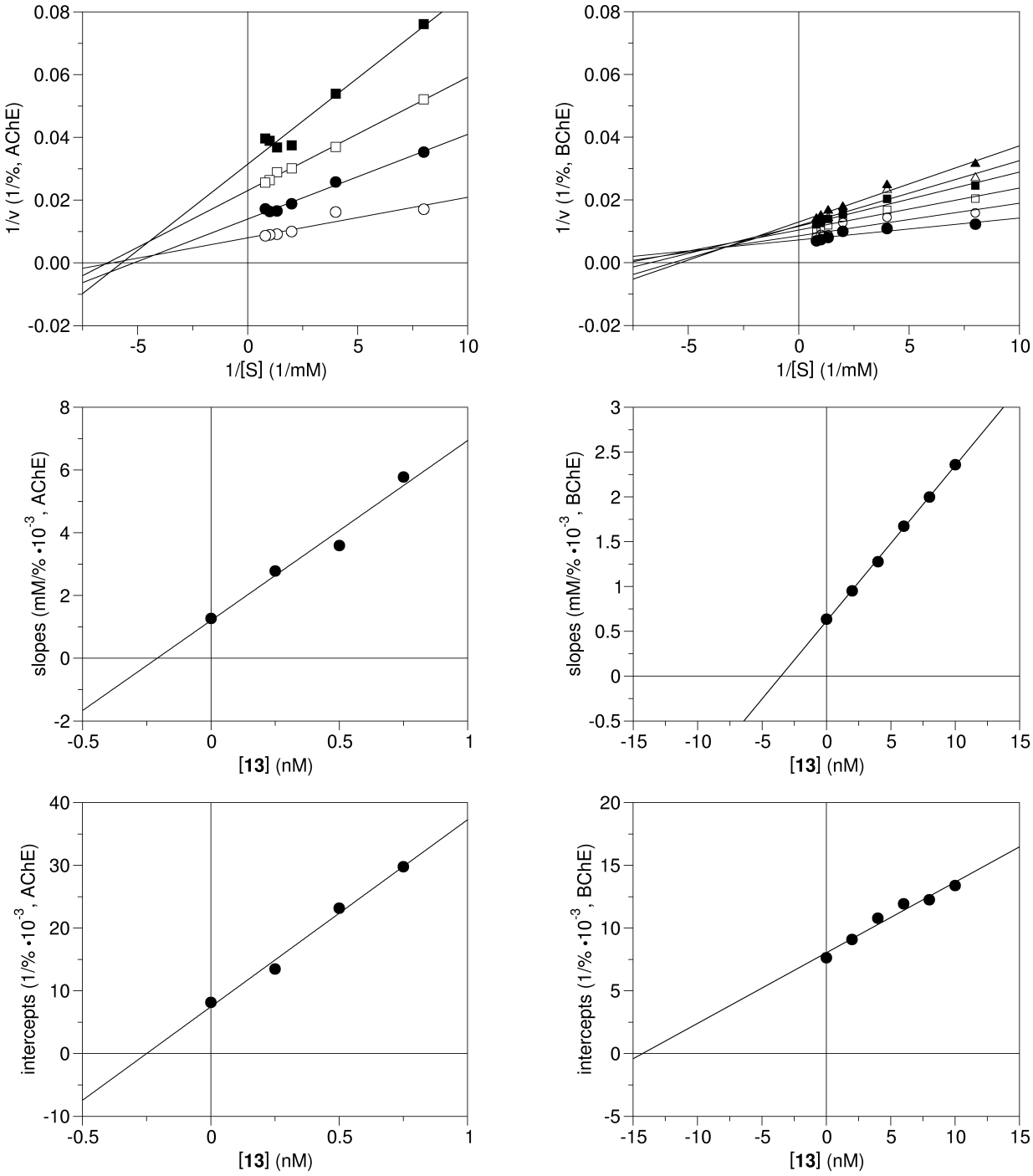
(4-Nitrophenyl)acetohydrazide (10). (4-Nitrophenyl)acetic acid (**9**; 20.0 mmol, 3.62 g) was dissolved in anhydrous ethanol (50 mL) and thionyl chloride (30.0 mmol, 2.2 mL) was added dropwise. The reaction mixture was stirred for 1 h at room temperature and subsequently evaporated. The remaining residue was redissolved in ethanol (5 mL) and heated to 80 °C in a mixture of hydrazine hydrate (100%, 10 mL) and water (15 mL). A precipitate formed and, after 45 min, the suspension was cooled to 0 °C to obtain **10** (3.62 g, 93%) by suction filtration, mp 169 °C, lit.[4] 167 °C. ^{1}H NMR (DMSO-d_6) δ 3.52 (s, 2 H), 4.24 (bs, 1 H), 7.52 (ddd, 2 H, *J* = 2.4, 2.6, 6.9 Hz), 8.16 (ddd, 2 H, *J* = 2.2, 2.5, 6.9 Hz), 9.28 (s, 1 H); ^{13}C NMR (DMSO-d_6) δ 40.27, 123.43, 130.41, 144.46, 146.44, 168.50. EA found: C, 49.5; H, 4.8, N, 21.4. $C_8H_9N_3O_3$ requires C, 49.2; H, 4.65, N, 21.5%.

2-(4-Nitrophenyl)-*N'*-1,2,3,4-tetrahydroacridin-9-ylacetohydrazide hydrochloride (11). A solution of 9-chloro-1,2,3,4-tetrahydroacridine (**8**; 20.0 mmol, 4.34 g) and (4-nitrophenyl)aceto-hydrazide (**10**; 20.0 mmol, 3.90 g) in anhydrous ethanol (150 mL) was heated to 140 °C in a sealed glass reactor. **11** (7.10 g, 86%) was afforded as a voluminous precipitate by suction filtration after 18 h, mp 277 °C (decomposition). ^{1}H NMR (DMSO-d_6) δ 1.71-1.87 (m, 4 H), 2.64 (t, 2 H, *J* = 5.7 Hz), 3.06 (t, 2 H, *J* = 5.9 Hz), 3.84 (s, 2 H), 7.49 (app. t, 1 H, *J* = 7.4 Hz), 7.60 (ddd, 2 H, *J* = 2.2, 2.5, 8.9 Hz), 7.85 (ddd, 1 H, *J* = 1.0, 7.1, 8.4 Hz), 7.97 (dd, 1 H, *J* = 0.9, 8.5 Hz), 8.19 (ddd, 2 H, *J* = 2.2, 2.5, 8.9 Hz), 8.59 (app. d, 1 H, *J* = 8.2 Hz), 9.69 (s, 1 H), 11.53 (s, 1 H), 14.36

(s, 1 H); ^{13}C NMR (DMSO-d_6) δ 20.24, 21.37, 24.21, 28.36, 39.53, 111.49, 115.16, 119.61, 123.51, 124.02, 125.90, 130.85, 133.00, 137.47, 142.94, 146.70, 152.95, 154.97, 168.75. EA found: C, 61.1; H, 5.4, N, 13.45. $C_{21}H_{21}ClN_4O_3$ requires C, 61.1; H, 5.1, N, 13.6%.

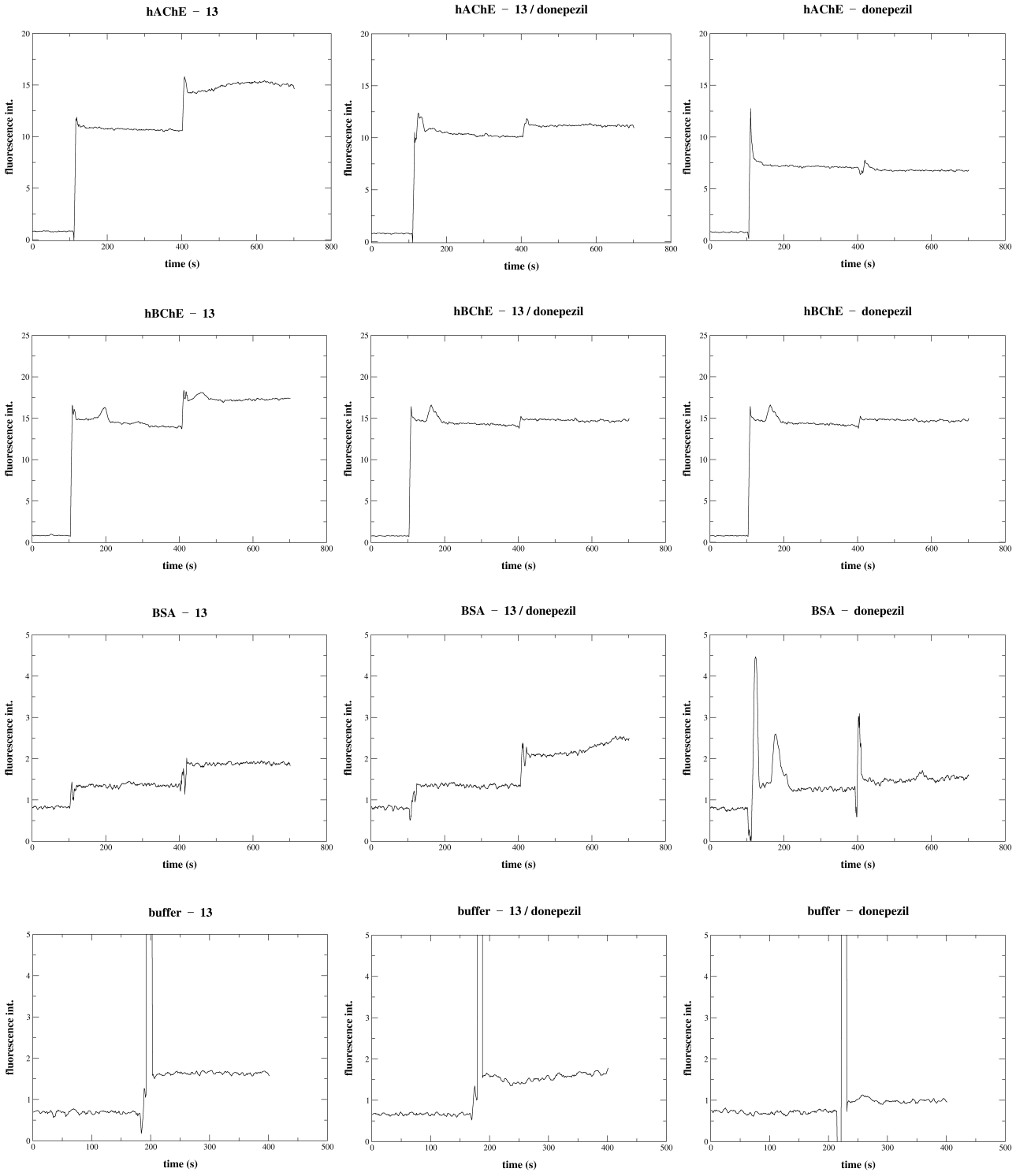
2-(4-Aminophenyl)-*N'*-1,2,3,4-tetrahydroacridin-9-ylacetohydrazide (12). Tin(II) chloride dihydrate (50 mmol, 11.28 g) was added to a supension of 2-(4-nitrophenyl)-*N'*-1,2,3,4-tetrahydroacridin-9-ylacetohydrazide hydrochloride (**11**; 10.0 mmol, 4.13 g) in anhydrous ethanol (100 mL) at 70 °C. The reaction mixture slowly turned clear and, after 2 h, undissolved material was filtered off. The filtrate was evaporated *in vacuo*, resuspended in water (100 mL) and the pH was adjusted to 10 by means of 1 M sodium hydroxide solution to be extracted with ethyl acetate (10 × 500 mL). The combined organic phases were dried using anhydrous sodium sulfate and evaporated *in vacuo* to obtain **12** (1.88 g, 54%) as a yellow powder, mp 195 °C (decomposition). ^{1}H NMR (DMSO-d_6) δ 1.67-1.83 (m, 4 H), 2.75 (t, 2 H, J = 6.0 Hz), 2.88 (t, 2 H, J = 6.2 Hz), 3.22 (s, 2 H), 4.86 (s, 2 H), 6.44 (ddd, 2 H, J = 2.4, 2.9, 8.2 Hz), 6.86 (ddd, 2 H, J = 2.4, 2.9, 8.2 Hz), 7.29 (ddd, 1 H, J = 1.3, 6.8, 8.4 Hz), 7.51 (ddd, 2 H, J = 1.3, 7.4, 8.3 Hz), 7.69 (dd, 1 H, J = 0.6, 8.4 Hz), 7.71 (s, 1 H), 8.33 (app. d, 1 H, J = 7.6 Hz), 10.17 (s, 1 H); ^{13}C NMR (DMSO-d_6) δ 22.45, 22.68, 24.86, 33.59, 39.53, 113.81, 115.51, 119.02, 122.40, 123.29, 123.46, 128.05, 129.65, 146.48, 147.30, 148.71, 157.81, 170.64. EA found: C, 70.5; H, 7.2, N, 14.5. $C_{21}H_{22}N_4O$ • EtOH requires C, 70.4; H, 7.2, N, 14.3%.

Lineweaver-Burk Analysis



Cholinesterase inhibition by **13** was analyzed at six substrate and four to six inhibitor concentrations in duplicate experiments. From Lineweaver-Burk analysis ([**13**] hAChE, pM: ○ 0, ● 250, □ 500, ■ 750; [**13**] hBChE, nM: ● 0, ○ 2, □ 4, ■ 6, ∆ 8, ▲ 10), K_{ic} and K_{iu} values were subsequently determined by plotting the respective slopes and intercepts *vs* the corresponding inhibitor concentrations.

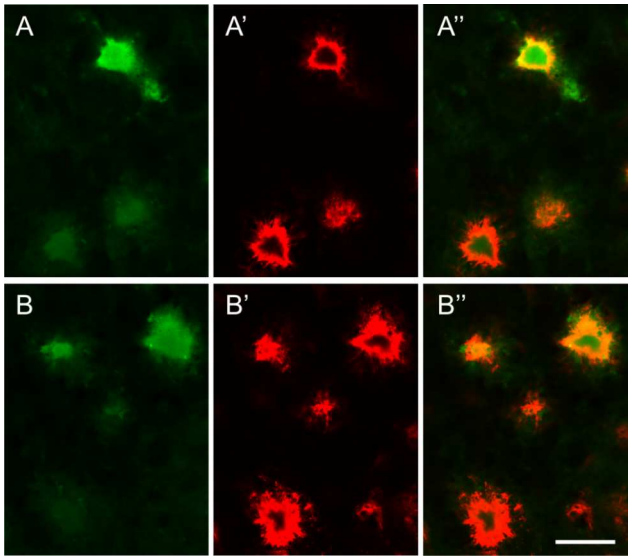
Fluorescence Experiments



Fluorescence intensities at (λ_{exc} = 405 nm and λ_{em} = 517 nm) of **13** measured in assay buffer with a final methanol concentration of 0.2%. *First line*: blank recording of assay buffer (930 µL); addition of a 3.66 µM hAChE solution (50 µL) after 100 s; addition of a 1 µM solution of **13** (20 µL), or a

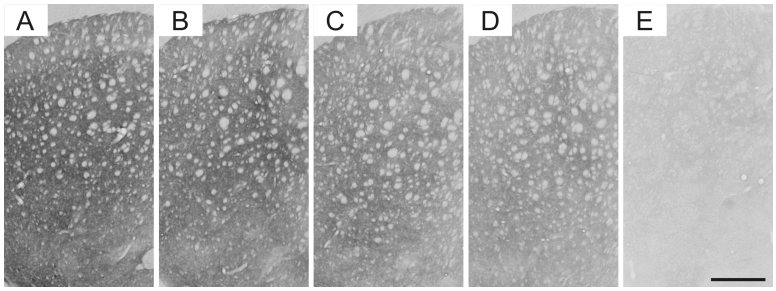
2 µM solution of **13** and a 400 µM solution of donepezil (each 10 µL), or a 200 µM solution of donepezil (20 µL) after 400 s. *Second line*: blank recording of assay buffer (930 µL); addition of a 3.66 µM hBChE solution (50 µL) after 100 s; addition of a 1 µM solution of **13** (20 µL), or a 2 µM solution of **13** and a 400 µM solution of donepezil (each 10 µL), or a 200 µM solution of donepezil (20 µL) after 400 s. *Third line*: blank recording of assay buffer (930 µL); addition of a 3.66 µM BSA solution (50 µL) after 100 s; addition of a 1 µM solution of **13** (20 µL), or a 2 µM solution of **13** and a 400 µM solution of donepezil (each 10 µL), or a 200 µM solution of donepezil (20 µL) after 400 s. *Fourth line*: blank recording assay buffer (980 µL); addition of a 1 µM solution of **13** (20 µL), or a 2 µM solution of **13** and a 400 µM solution of donepezil (each 10 µL), or a 200 µM solution of donepezil (20 µL) after 200 s.

Labeling of Senile Plaques with 13 in the Presence and Absence of Fasciculin-2



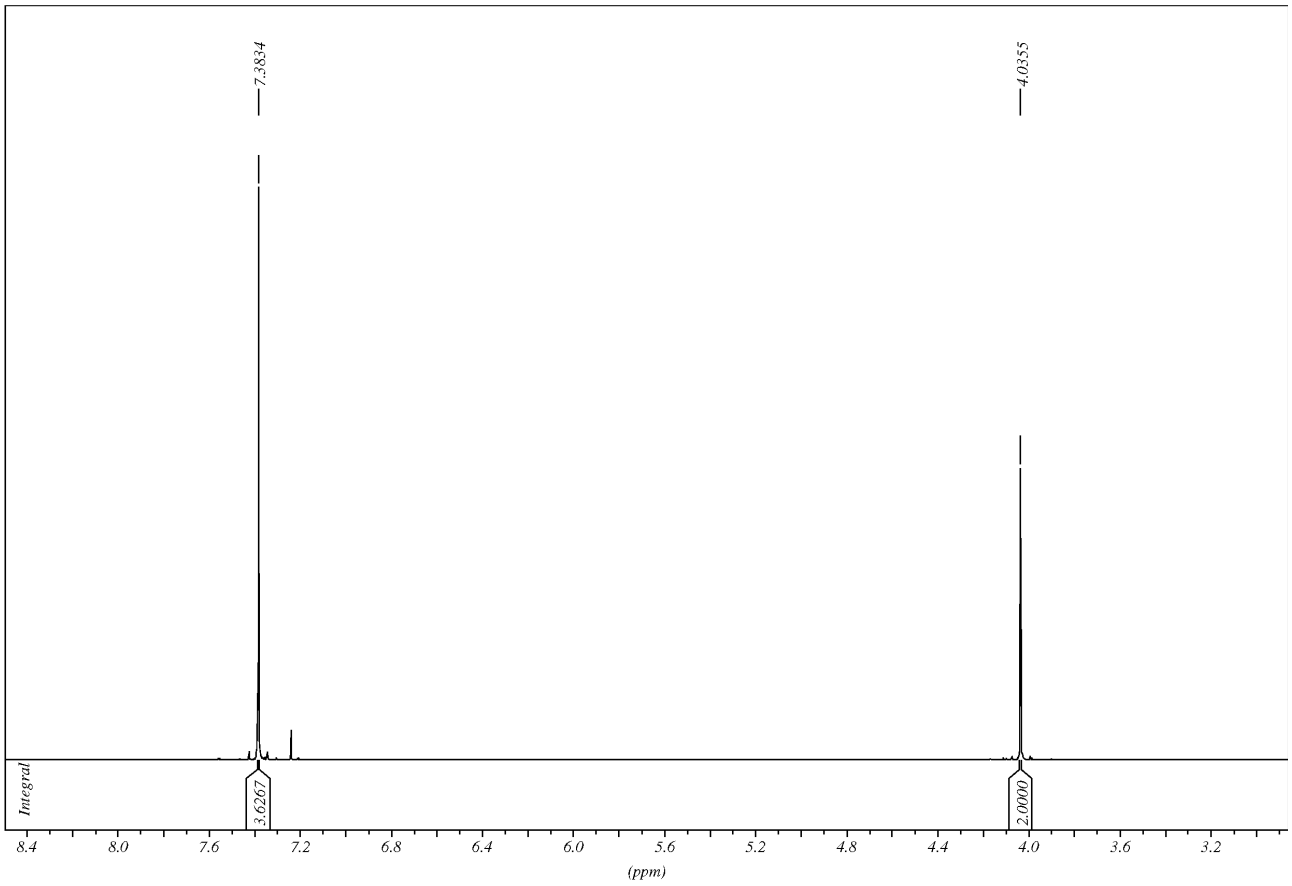
Double fluorescence labeling of senile plaques in the hippocampus from a 16-months-old triple-transgenic mouse with age-dependent β-amyloidosis. Confocal laser scanning microscopy reveals the combined staining with 6.8 µM **13** (A, B) and Aβ-immunodetection of the same tissue based on rabbit-anti-β-amyloid and red fluorescent carbocyanine 3-conjugated anti-rabbit IgG (A', B') after pretreatment of tissue sections with 3.1 µM fasciculin-2 (A–A'') or without pretreatment (B–B''). The allocation of both markers in the same plaques becomes obvious in the merged Figures A'' and B'' (scale bar = 25 µm).

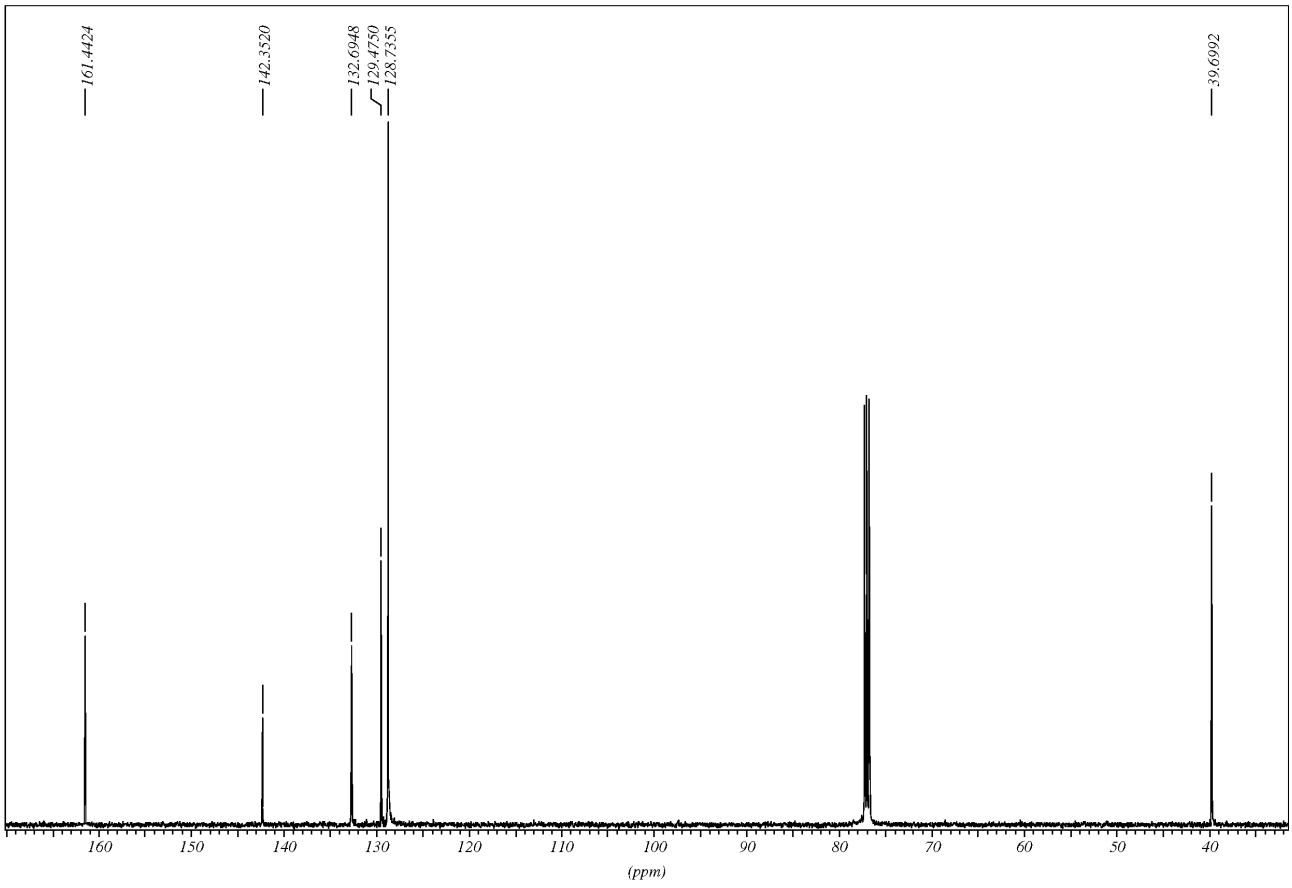
Histochemical Enzyme Activity Assay with Different Concentrations of 13



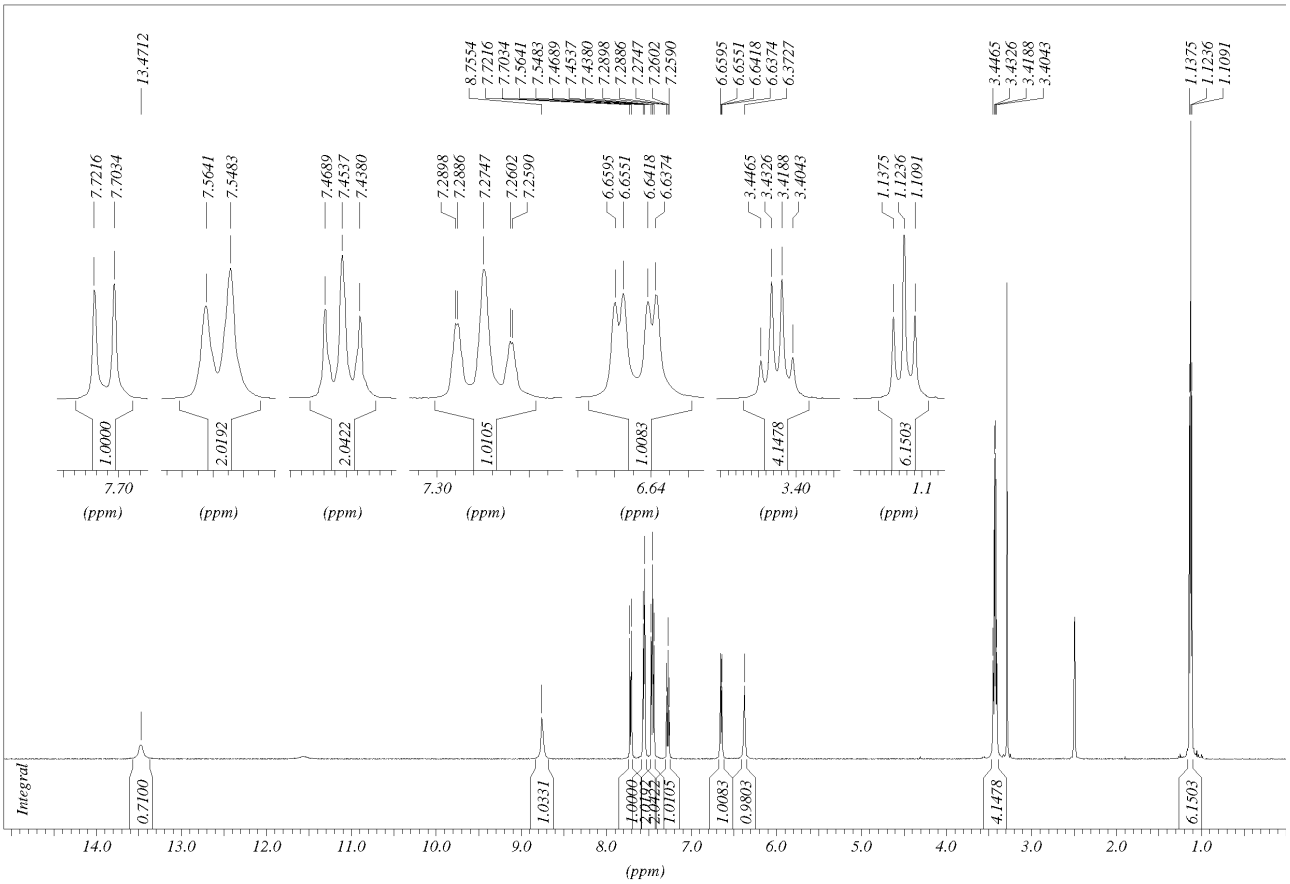
Histochemically detected AChE inhibition by **13** shows a concentration-dependent manner. Staining of AChE activity (30 min, 37 °C) in the dorsal striatum of a triple-transgenic mouse without inhibitor pretreatment (A), and after pretreatment with **13**: 68 nM (B), 680 nM (C), 6.8 µM (D) and 68 µM (E) (scale bar = 500 µm).

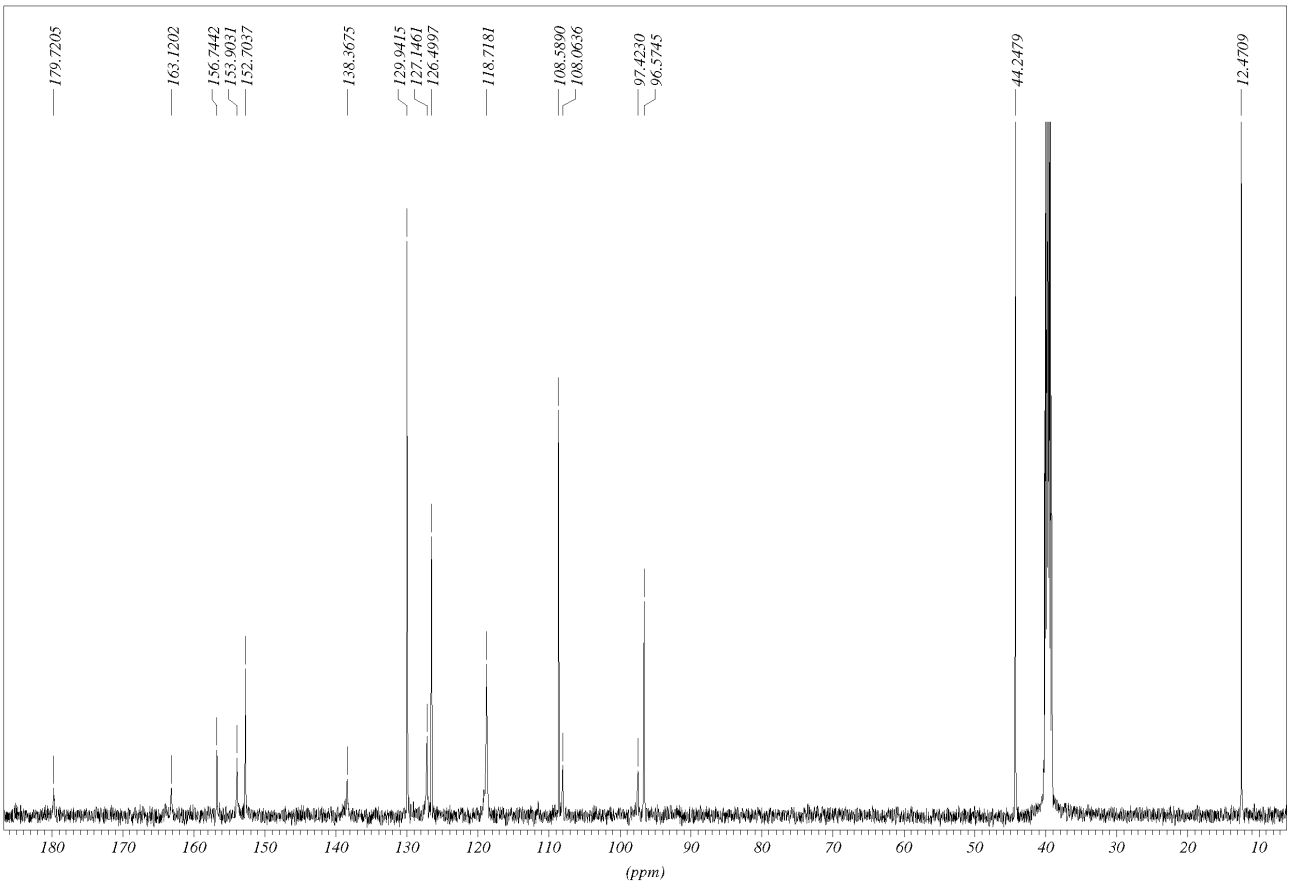
NMR spectra of 3



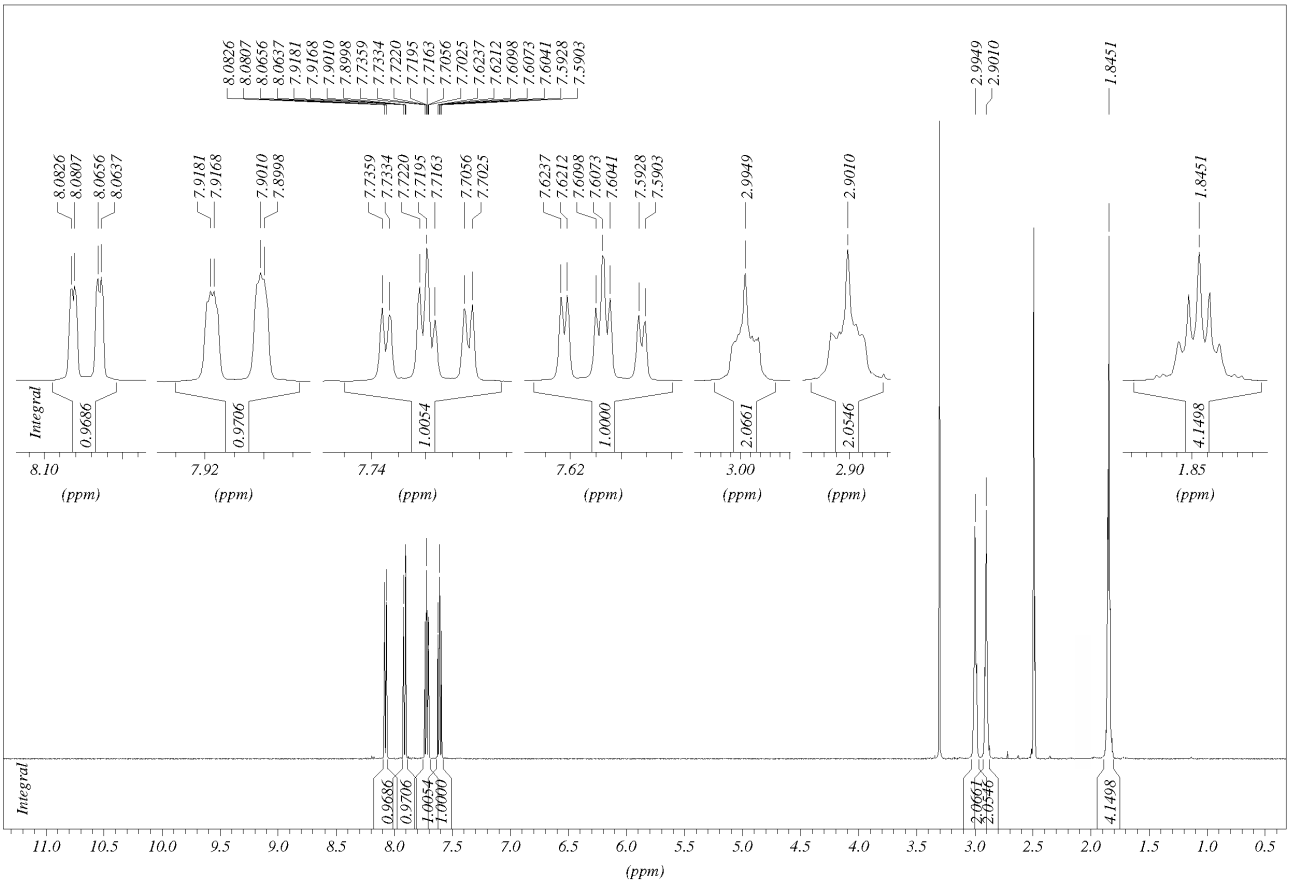


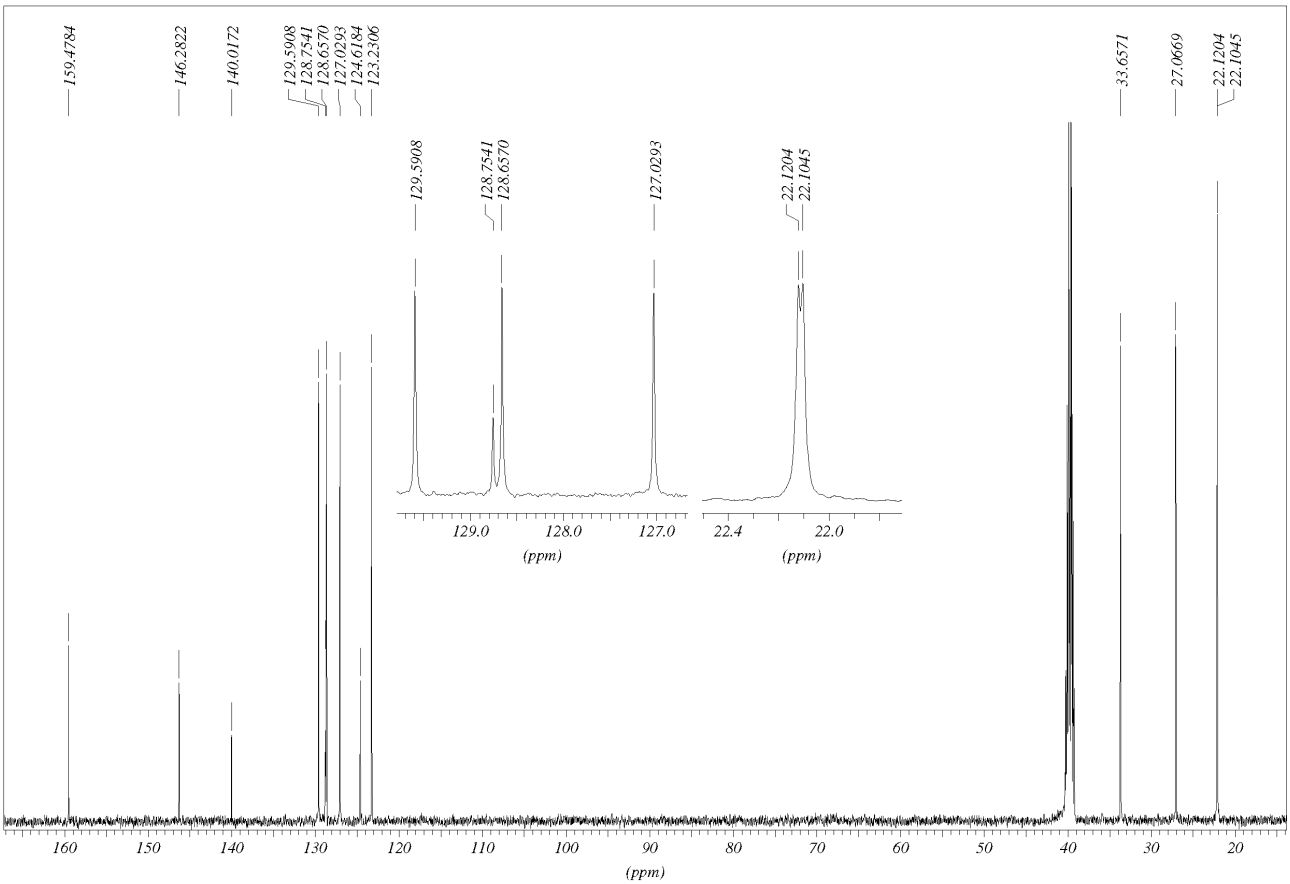
NMR spectra of 5



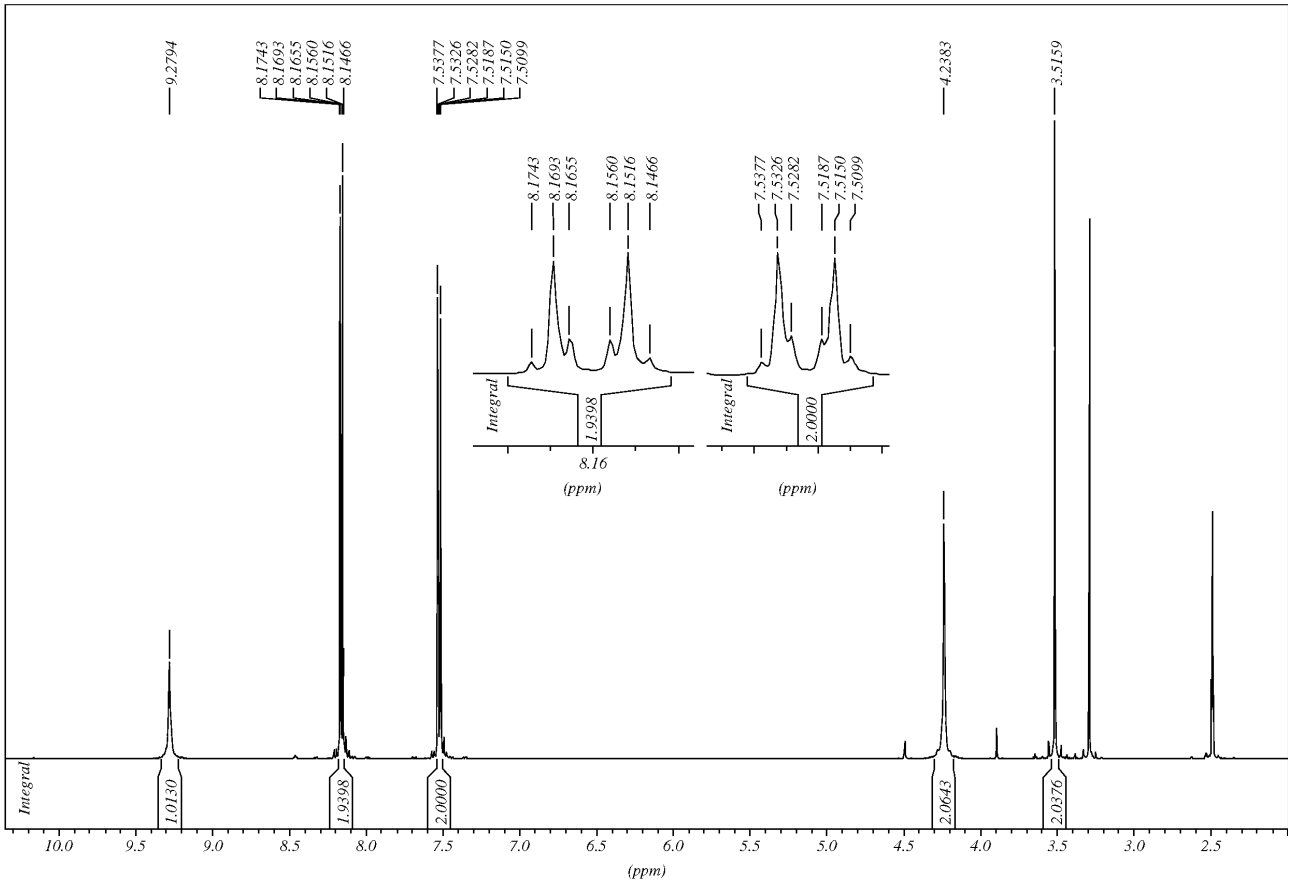


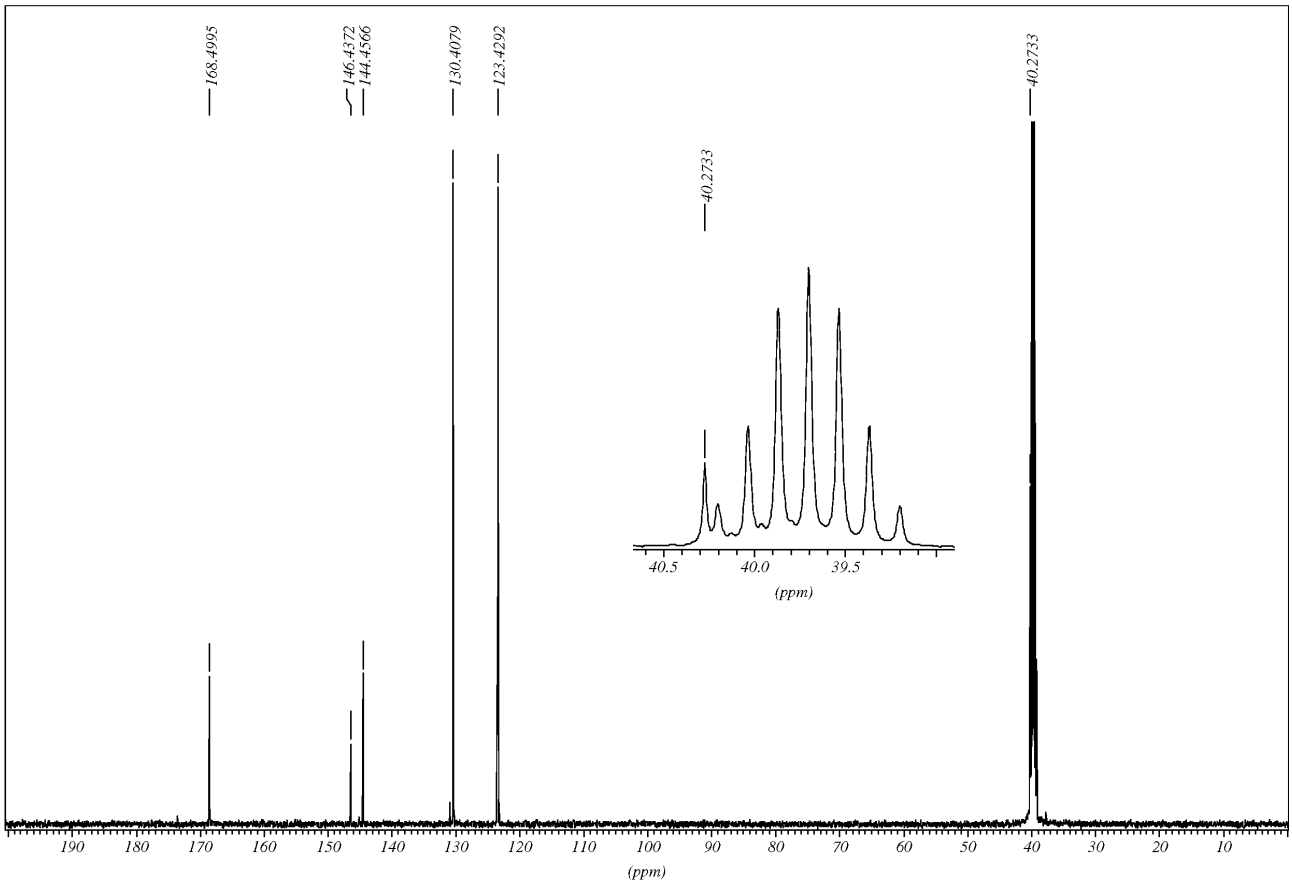
NMR spectra of 8



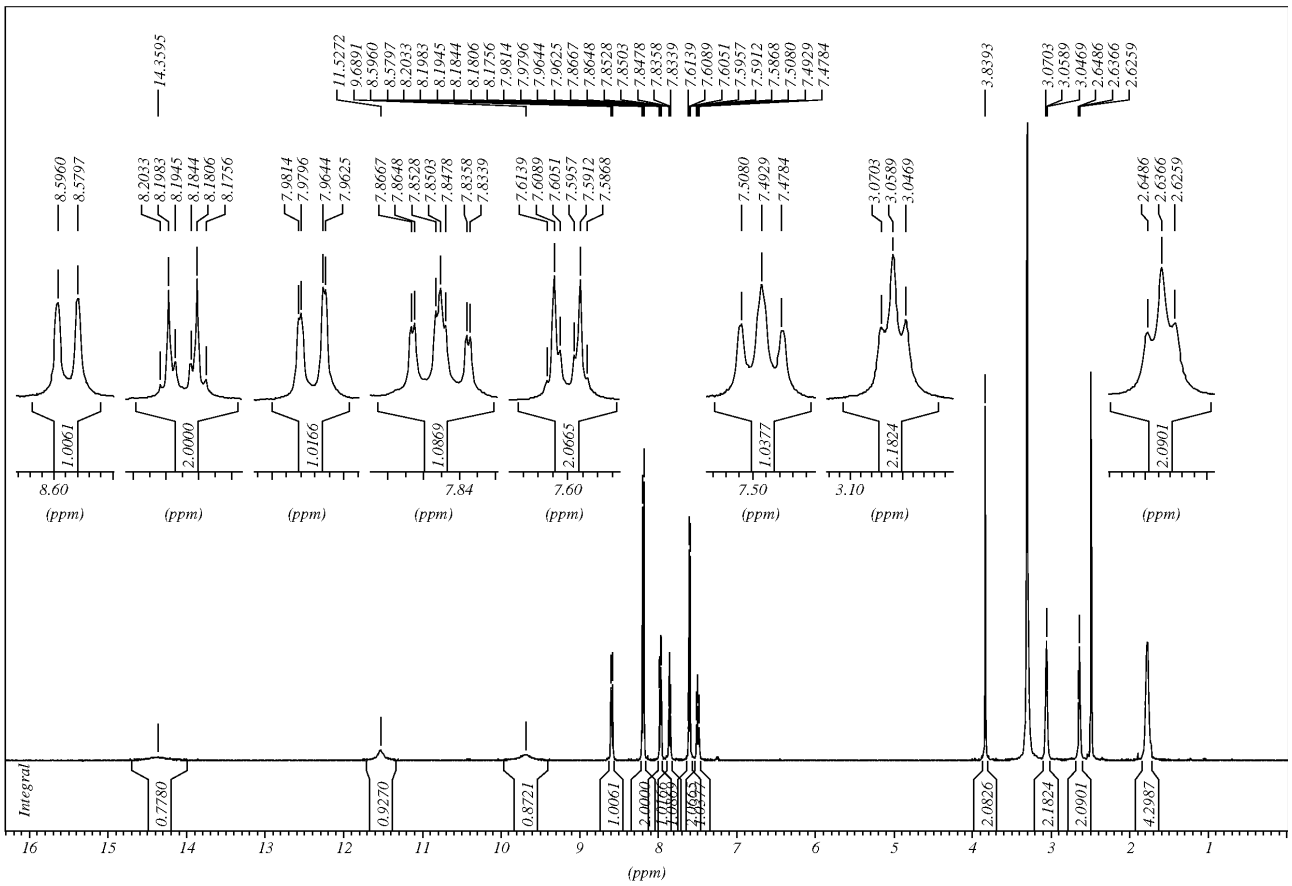


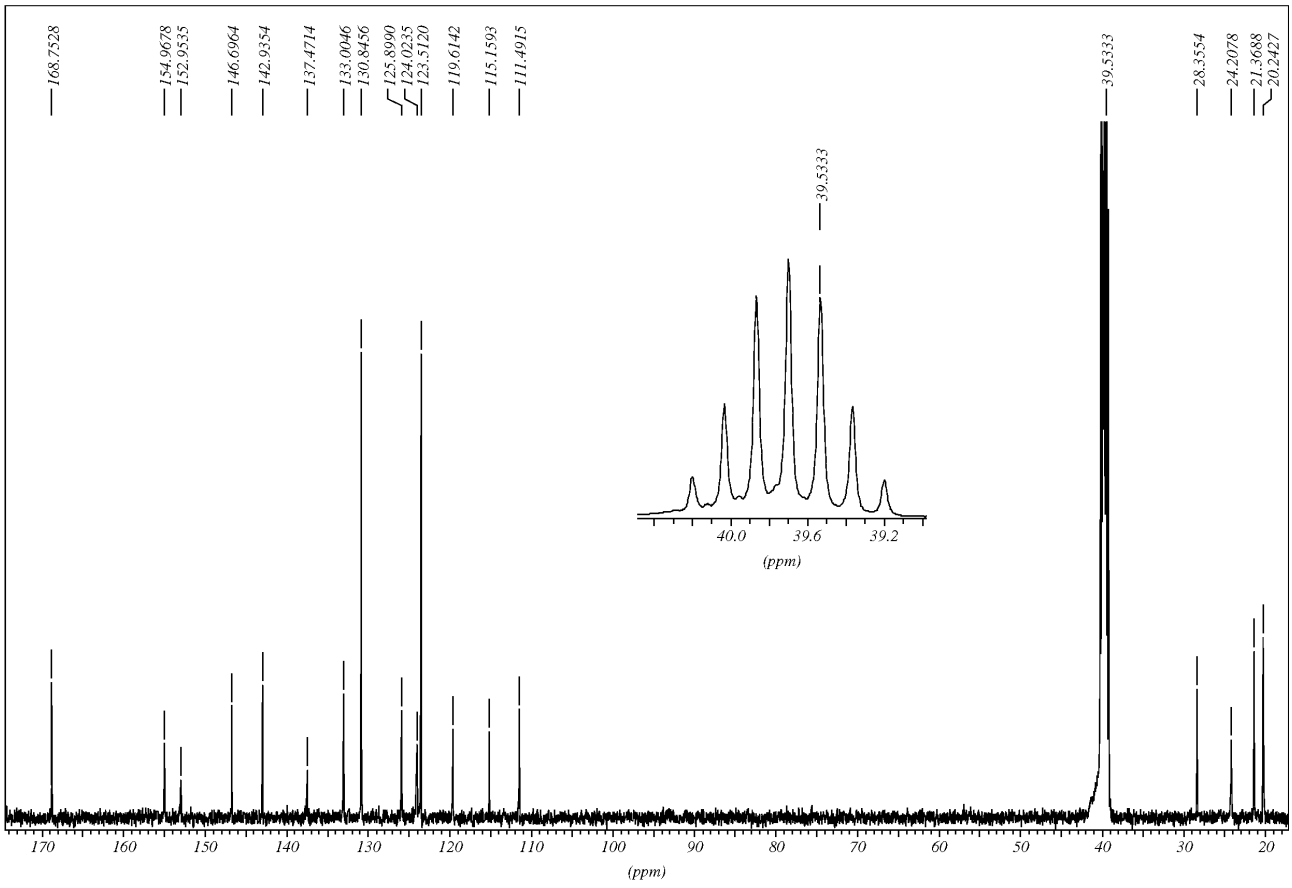
NMR spectra of 10



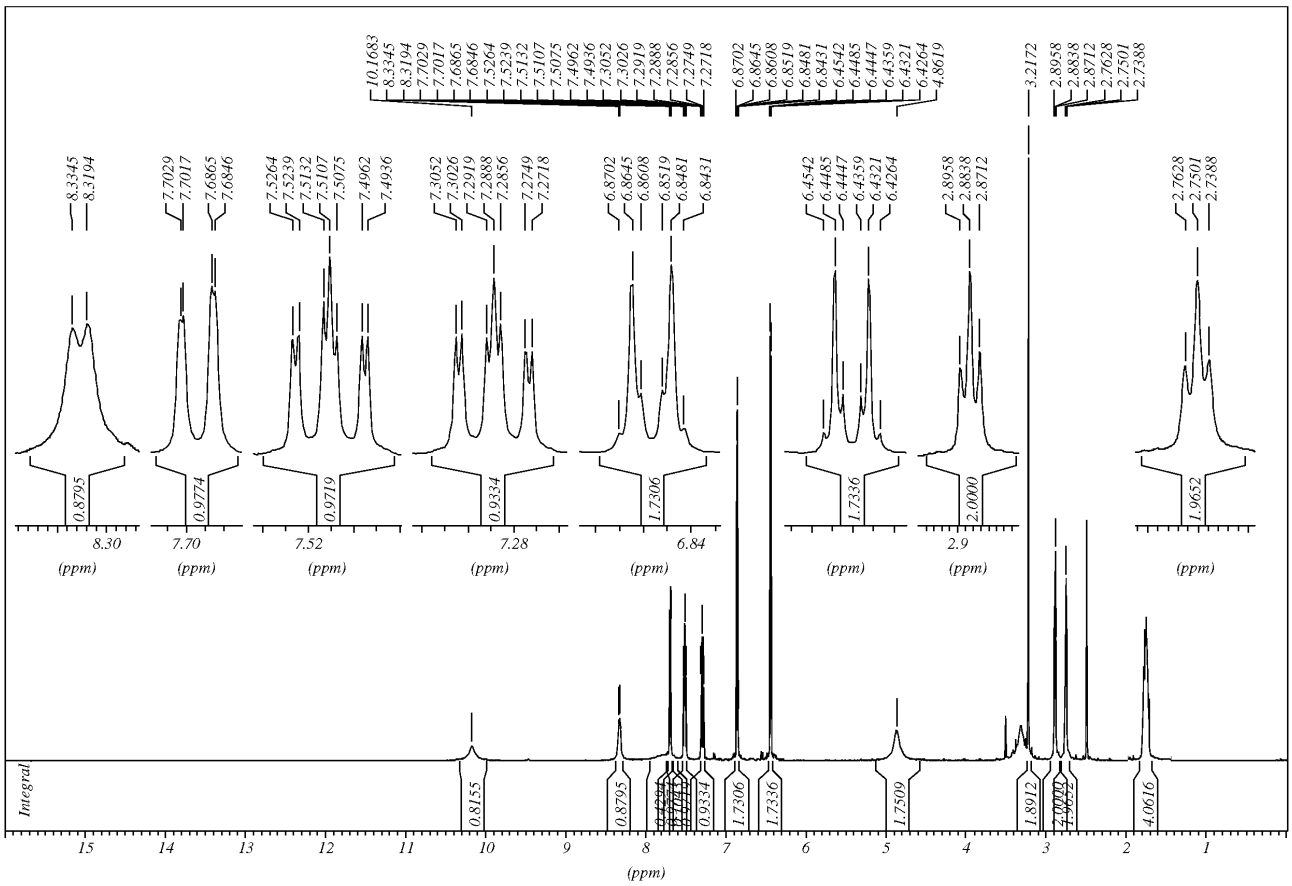


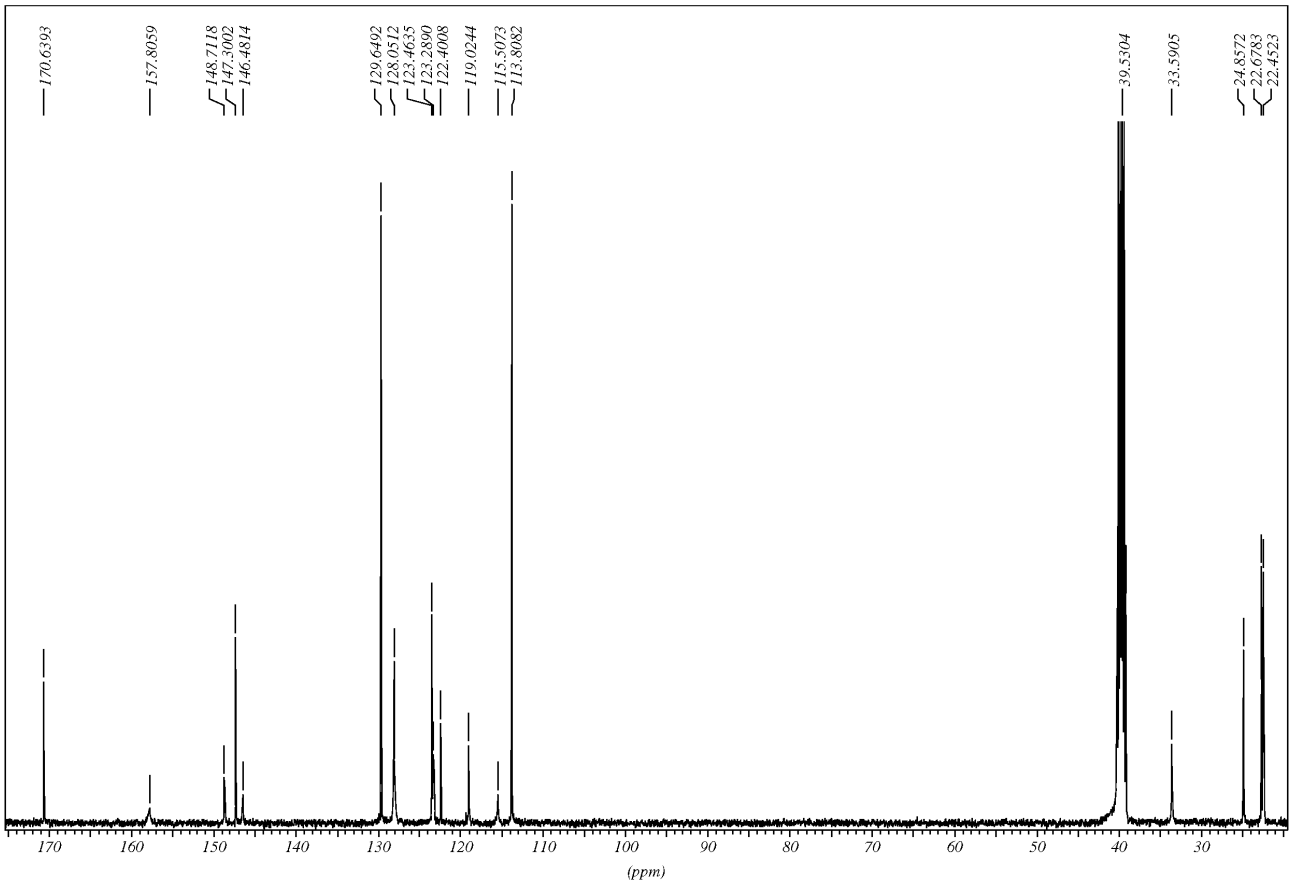
NMR spectra of 11



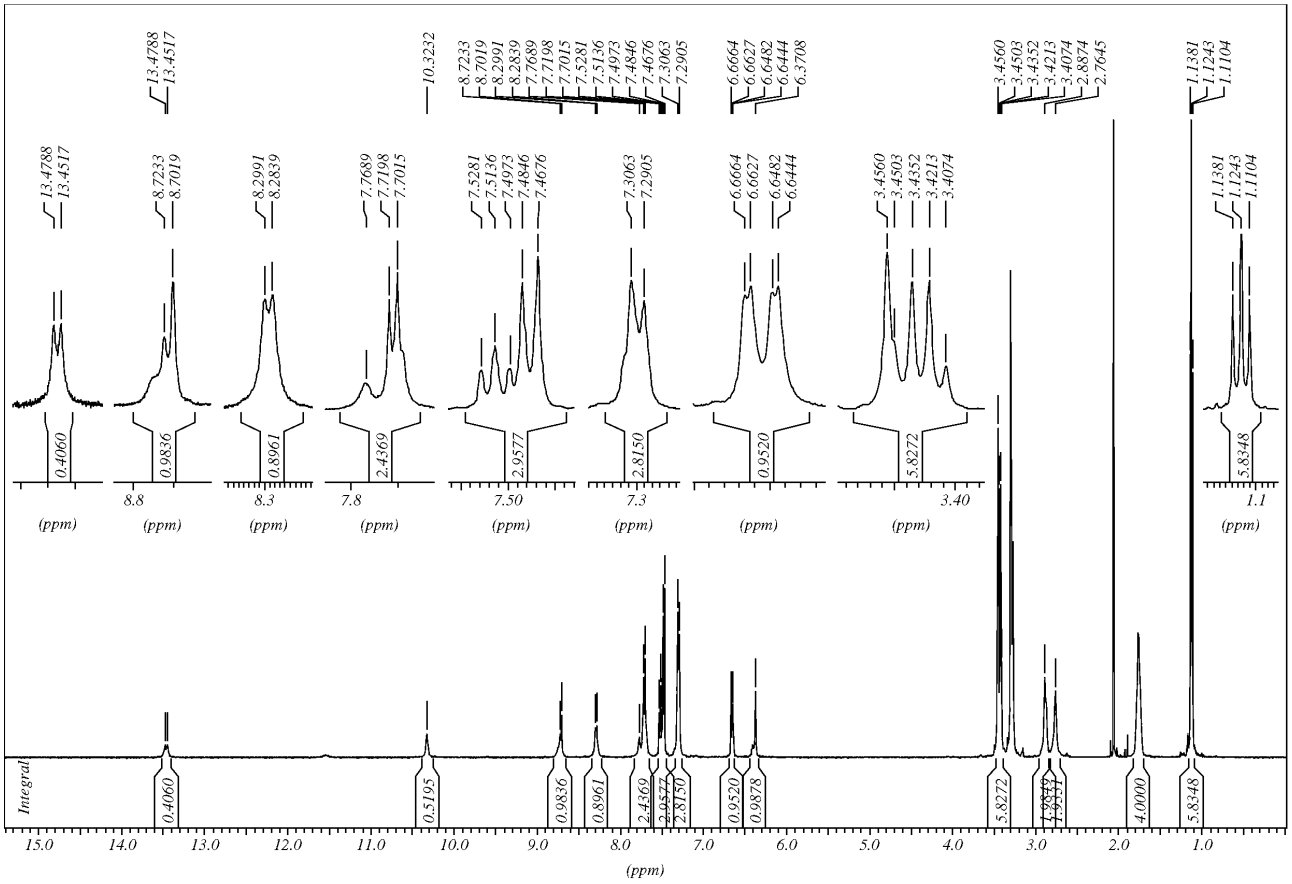


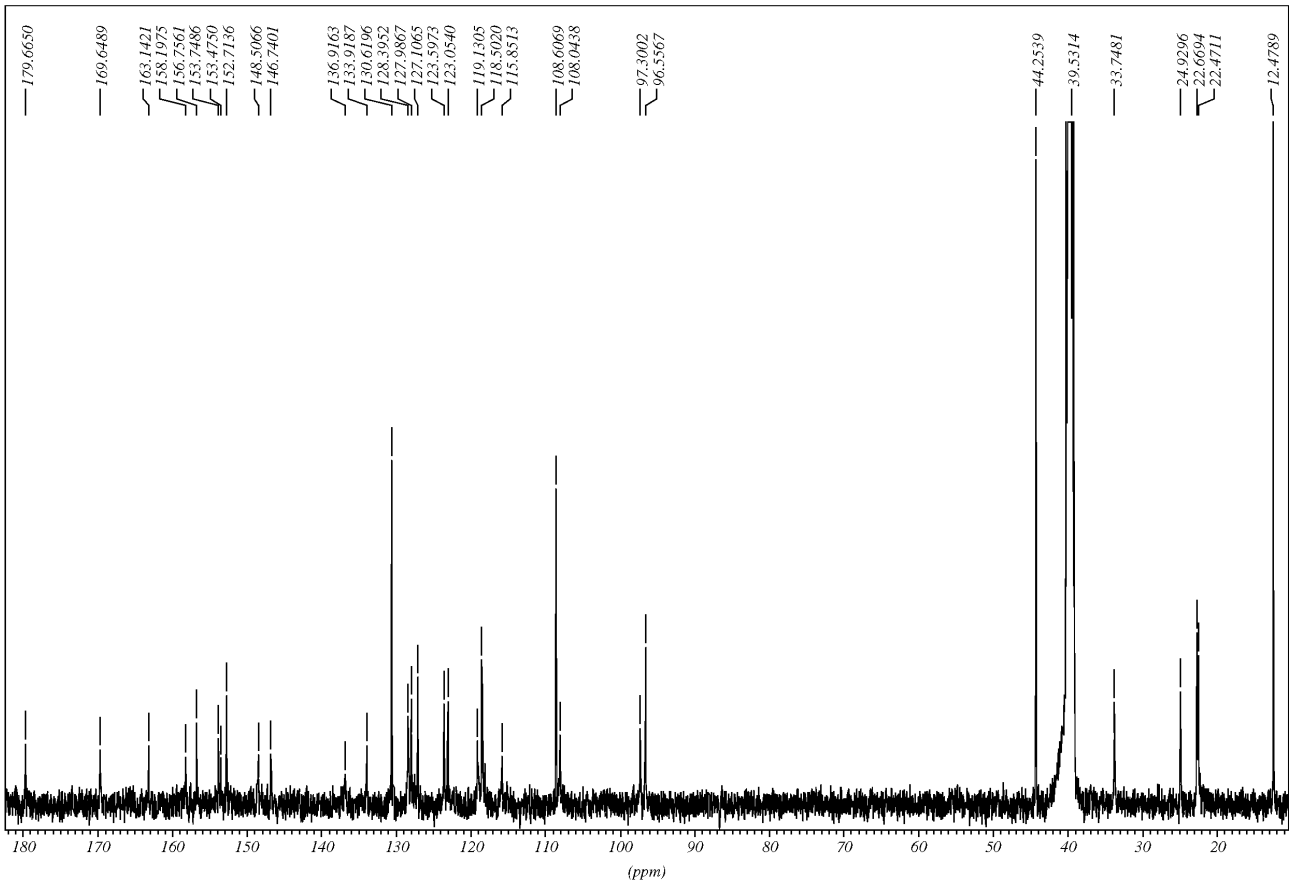
NMR spectra of 12



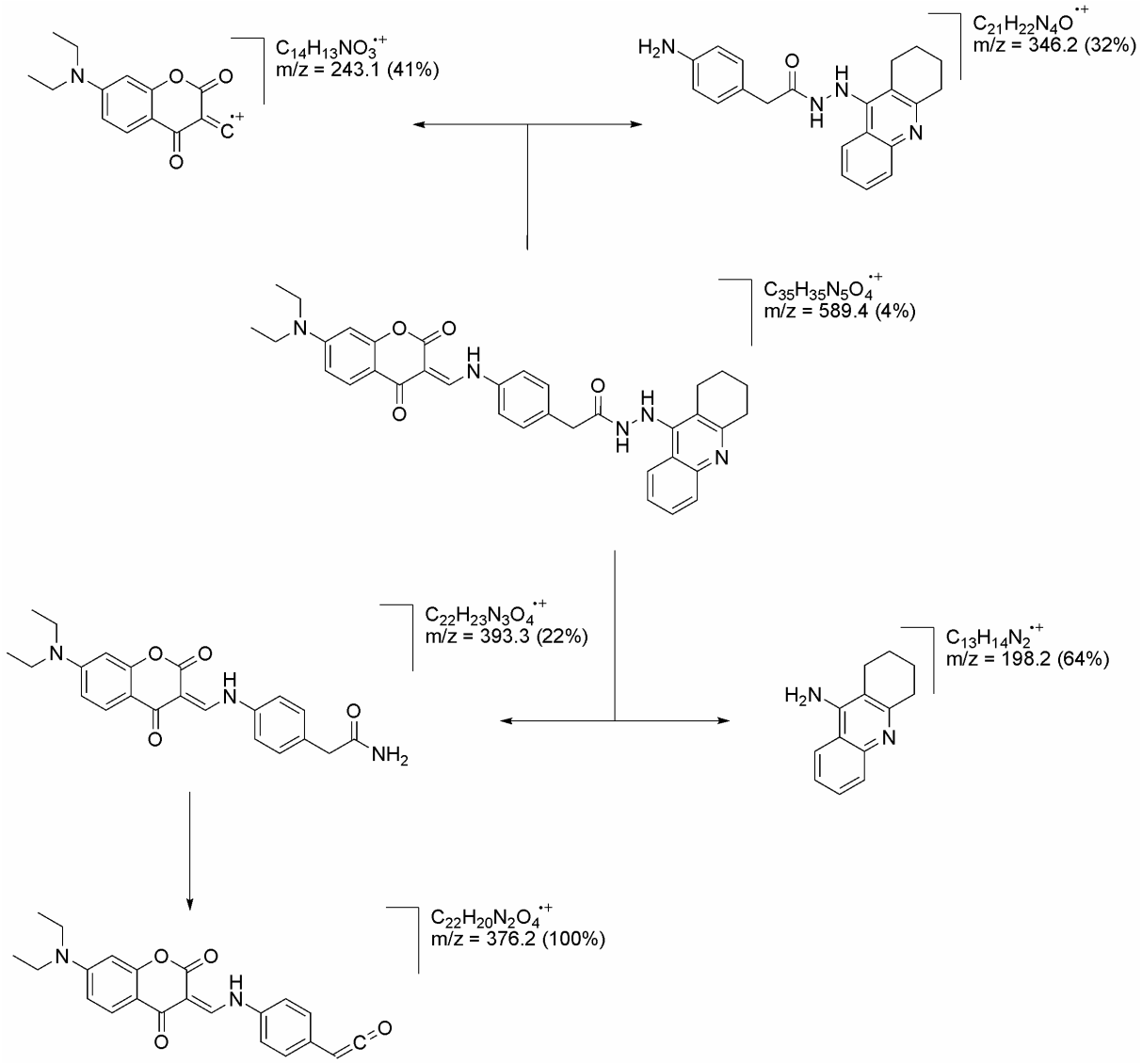


NMR spectra of 13





Mass Spectrometry Analysis of 13



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