Supporting Information

Site-Activated Chelators Targeting Acetylcholinesterase and Monoamine Oxidase for Alzheimer's Therapy

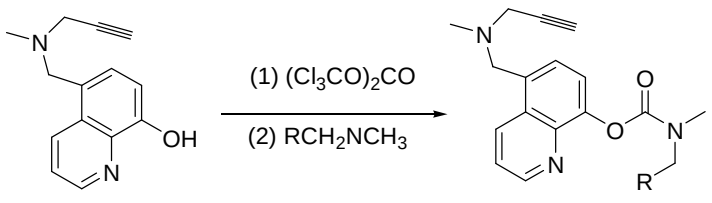
Hailin Zheng[a,b], Moussa B.H. Youdim[c,1] and Mati Fridkin[a,1]

Table of Contents:

1. Chemistry

1.1. General: Starting materials for chemical synthesis were obtained from the following companies: Aldrich (USA), Merck (Germany), Fluka (Switzerland). HPLC grade acetonitrile and trifluoracetic acid (TFA) were from JT Baker (Phillipsburg, NJ). Tetrahydrofuran (THF) was distilled over $LiAlH_4$ and passed through an Al_2O_3 column. NMR spectra were measured on a Bruker WH-270, a Bruker DPX-250, or a Bruker AMX-400 NMR spectrometer. Flash column chromatography separations were performed on silica gel Merck 60 (230-400 mesh ASTM). UV/VIS spectra were measured on a Hewlett-Packard 8450A diode array spectrophotometer. TLC was performed on E. Merck Kieselgel 60 F_{254} plates. Staining of TLC plates was done by basic aqueous 1% $KMnO_4$. Mass spectra (DI, EI-MS) were measured on a VG-platform-II electrospry single quadropole mass spectrometry (Micro Mass, UK). The purifications were performed on a Spectra-Physics SP8800 HPLC system equipped with an Applied Biosystems 757 variable wavelength absorbance detector that was set to 254 nm. HPLC pre-packed columns were Vydac RP-18 column (250 ×22 mm; 10 μm bead size, Merck, Darmstadt, Germany) for preparative purifications and an RP-18, 100 mm × 4.6 mm Chromolith column (Merck) for purity determinations. All new compounds for biological studies have purity not less than 95%. The purification was achieved by using a linear gradient comprising 0.1% TFA in H_2O (solution A) and 0.1% TFA in 75% aqueous acetonitrile (solution B) with a flow of 10.0 mL/min over 50 min. The purified products were neutralized by 5% $NaHCO_3$ and extracted with dichloromethane or ethyl acetate to afford products as free base. Starting material **M30** was prepared as described previously (1).

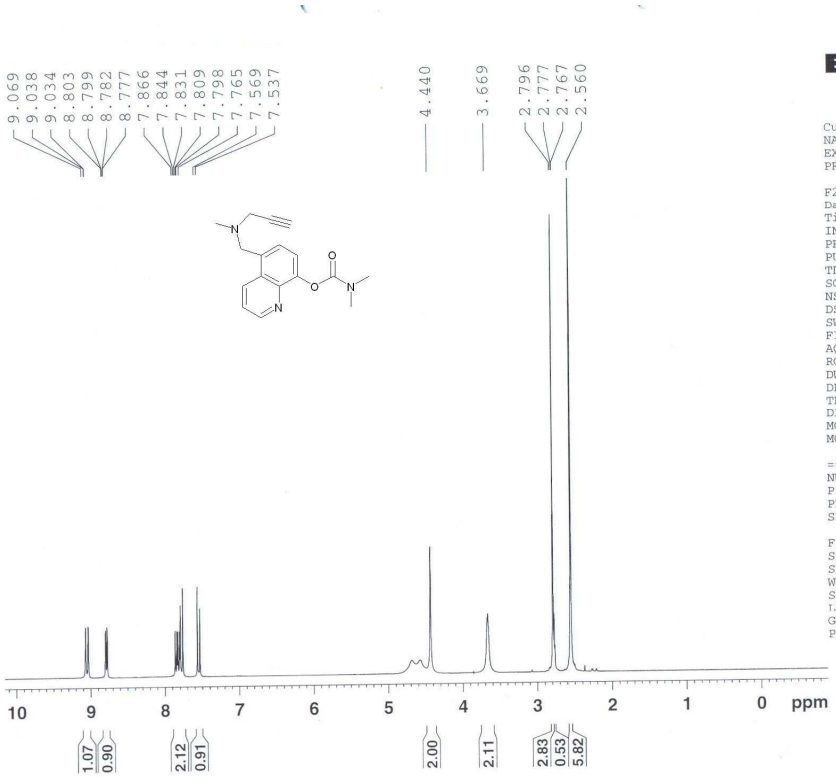
1.2. Synthesis of new prochelators 2 and 3



M30

2: R = H; 3: **R** = CH_3

Synthesis of 5-(N-methyl-N-propargylaminomethyl) quinolin-8-yl dimethyl carbamate 2 . To a stirred solution of **M30** (45 mg, 0.2 mmol), pyridine (97 μL, 1.2 mmol in anhydrous THF (1 mL) was added triphosgene (71 mg, 0.24 mmol) under argon atmosphere at 0 ºC. After 0.5 h stirring at 0 ºC, dimethylamine (1 N solution in THF) (0.24 mL ,0.22 mmol) was added, and the reaction mixture was stirred for 0.5 h at 0 ºC and then 0.5 h at room temperature. After evaporation of the solvent, water was added and the resulting mixture was extracted with ethyl acetate. The combined organic extracts were washed with brine and dried with $NaSO_4$. Evaporation of the solvent gave a crude product that was purified by RP HPLC and characterized by LC-MS, H^1 NMR, and ^{13}C NMR. (38 mg, 64 % yield, purity 97%). H^1 NMR (250 MHz,D_2O), 2.56 (s, 6H), 2.77 (m, 1H), 2.80 (s, 3H), 3.67 (s, 2H), 4.44 (s, 2H), 7.55 (d, J = 8.0 Hz, 1H), 7.80 (m, 2H), 8.79 (d, J=5.50 Hz, 1H), 9.06 (d, J=8.75 Hz, 1H). ^{13}C NMR (63 MHz, hydrochloric salt in D2O) 39.46, 39.51, 42.91, 47.98, 56.31, 74.14, 84.09, 126.33, 127.30, 129.33, 131.91, 134.95, 138.10, 146.04, 146.53, 148.35, 157.21. Mass Mass spectrometry: calculated for $C_{20}H_{23}N_3O2$ m/z $[M+H]^+$ =298.35, found $[M+H]^+$ = 298.53.



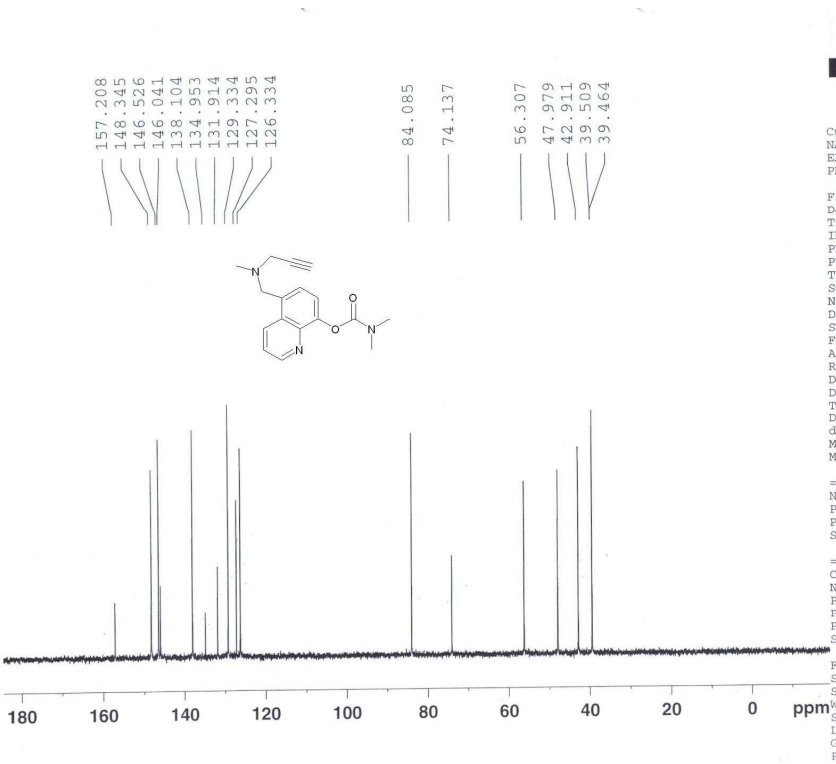
BRUKER

Current Data Parameters
NAME H358 HPLC
EXPNO 1
PROCNO 1

F2 - Acquisition Parameters
Date_ 20060902
Time 10.43
INSTRUM spect
PROBHD 5 mm QNP 1H/1
PULPROG zg
TD 16384
SOLVENT D2O
NS 30
DS 0
SWH 4990.020 Hz
FIDRES 0.304567 Hz
AQ 1.6417269 sec
RG 45.3
DW 100.200 usec
DE 6.00 usec
TE 300.0 K
D1 1.00000000 sec
MCREST 0.00000000 sec
MCWRK 0.01500000 sec

======== CHANNEL f1 ========
NUC1 1H
P1 10.50 usec
PL1 -6.00 dB
SFO1 250.1310403 MHz

F2 - Processing parameters
SI 32768
SF 250.1300754 MHz
WDW EM
SSB 0
LB 0.01 Hz
GB 0
PC 1.00



BRUKER

Current Data Parameters
NAME H358 C HPL
EXPNO 2
PROCNO 1

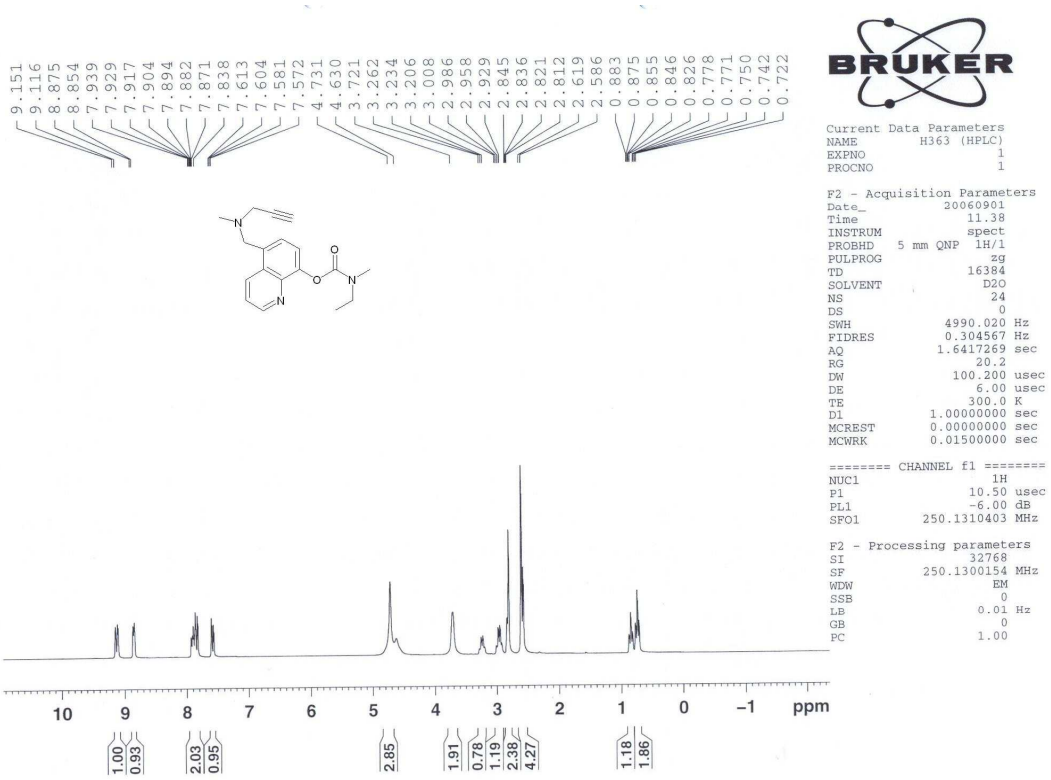
F2 - Acquisition Parameters
Date_ 20060902
Time 11.04
INSTRUM spect
PROBHD 5 mm QNP 1H/1
PULPROG zgdc
TD 65536
SOLVENT D2O
NS 311
DS 0
SWH 18832.393 Hz
FIDRES 0.287360 Hz
AQ 1.7400308 sec
RG 11585.2
DW 26.550 usec
DE 6.00 usec
TE 300.0 K
D1 2.00000000 sec
d11 0.03000000 sec
MCREST 0.00000000 sec
MCWRK 0.01500000 sec

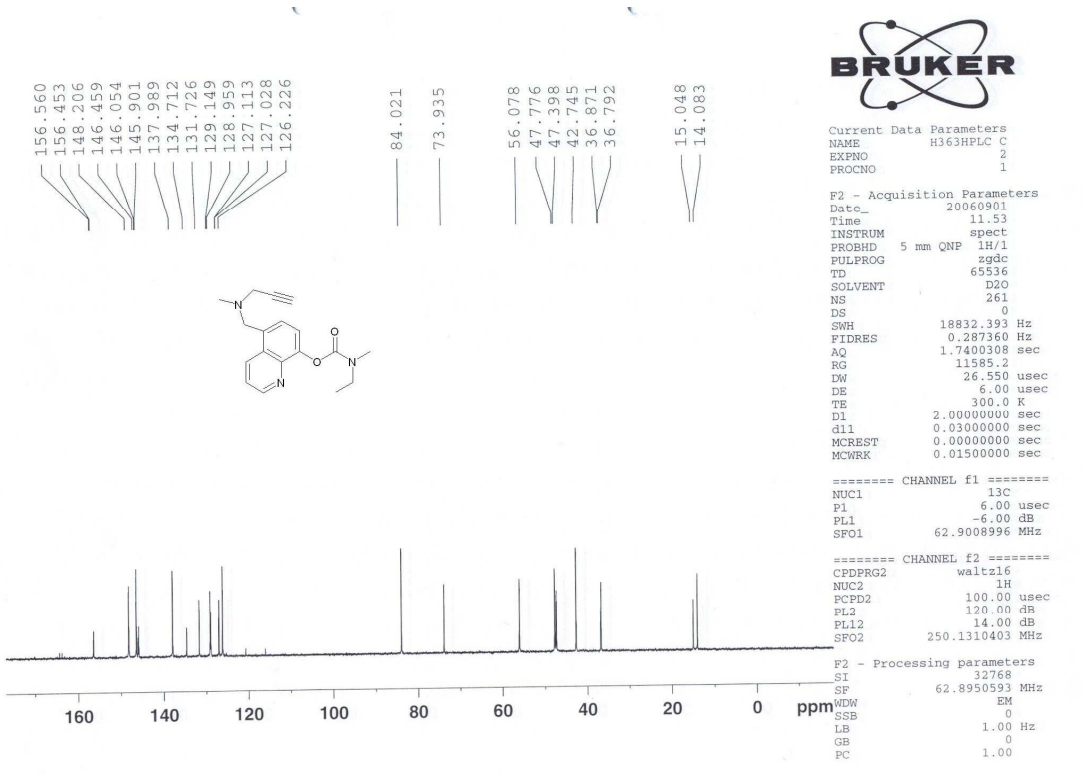
======== CHANNEL f1 ========
NUC1 13C
P1 6.00 usec
PL1 -6.00 dB
SFO1 62.9008996 MHz

======== CHANNEL f2 ========
CPDPRG2 waltz16
NUC2 1H
PCPD2 100.00 usec
PL2 120.00 dB
PL12 14.00 dB
SFO2 250.1310403 MHz

F2 - Processing parameters
SI 32768
SF 62.8950593 MHz
WDW EM
SSB 0
LB 1.00 Hz
GB 0
PC 1.00

Synthesis of 5-(N-methyl-N-propargylaminomethyl) quinolin-8-yl ethylmethyl carbamate 3. 3 was synthesyzed by the method as described above for synthesis of **2**. Yield: 60%, purity 98%. H^1 NMR (250 MHz,D_2O), 0.80 (m, 3H), 2.57 (s 1H), 2.62 (s, 3H), 2.83 (s, 3H), 3.10 (m, 2H), 3.72 (s, 3H), 4.73 (s, 3H), 7.59 (d, J = 8.0 Hz, 1H), 7.90 (m, 2H), 8.86 (d, J=5.25 Hz, 1H), 9.13 (d, J=8.75 Hz, 1H). ^{13}C NMR (63 MHz, hydrochloric salt in D2O) 14.08, 15.05, 36.83, 42.75, 47.40, 47.78, 56.08, 73.94, 84.02, 126.23, 127.03, 129.15, 131.73, 134.71, 137.99, 146.46, 148.21, 156.45. Mass spectrometry: calculated for $C_{18}H_{21}N_3O_2$ m/z $[M + Na]^+$ =334.38, found $[M +Na]^+$ = 334.51.





2. Biology

2.1. Determination of inhibition of AChE and BChE activity: Ellman's method was adapted for determination of AChE and BuAChE activities in rat brain homogenates. Brain tissue from adult Wistar rats was homogenized at 2% w/v (or 10%, for butyrylcholinesterase) in 0.1 M sodium phosphate buffer, pH 7.4, with added NaCl 58.5 g/L and Triton X-100 0.05% v/v. Aliquots of homogenate (20 μL) were incubated with HLA20A for designed time intervals in phosphate buffer pH 7.4 before addition of 5,5′-dithiobis(2-nitrobenzoic) acid and either 1 mM acetylthiocholine iodide or 10 mM butyrylthiocholine iodide (Sigma, St. Louis, MO). The reaction was run at 37 °C in a final volume of 200 μL in 96-well microplates and was followed at 412 nm for 5 min with a plate reader. In every experiment, cholinesterase-independent (nonspecific) substrate hydrolysis was determined by including one experimental group treated with tacrine 30 μM; appropriate tissue and reagent blanks were also included. Reaction velocities were determined in three or four replicates per condition; these were averaged and expressed as percent activity relative to control (solvent), after subtracting the rate of

nonspecific hydrolysis. Results are reported as mean (SEM of IC_{50} obtained independently from three to five experiments).

2.2. Cytotoxicity Assays: Human SH-SY5Y neuroblastoma cells were plated in 100-mm culture dishes and cultured in DMEM (4,500 mg/l glucose), containing 10% FCS (fetal calf serum, Bet Haemek, Israel), and 1% of a mixture of penicillin/ streptomycin/ nystatin. When cells reached the required confluence, the culture medium was removed and the cells were detached by vigorous washing followed by centrifugation at 200 g for 5 min. the cells (0.5 × 104 cells/well) in DMEM with 10% of fetal calf serum, were placed in microtiter plates (96 wells) precoated with collagen (10 mg/cm2). The cells were allowed to attach for 24 h before drugs were added. The cell viability was tested by the 3-(4.5-dimethylthiazol-2-yl)-2.5-diphenyltetrazolium (MTT) assays (see below) after 48 h incubation.

2.3. MTT Test: The MTT test is based on the conversion of MTT to blue formasan crystals by viable cells. Briefly, 24 h after treatment, the medium was removed and replaced with a medium (100 μl/well) lacking serum. To each well, 10 μl of a 5 mg/ml MTT solution in PBS was added. After incubation at 37 ºC for 2 h, 100 μl of 10% SDS in 0.01N HCl was added and the solution was mixed thoroughly and incubated for additional 24 h. Absorption was determined in a Perkin-Elmer Dual Wave-length Eliza-Reader at l = 570 nm/650 nm after automatic subtraction of background readings. Cell viability was expressed as a percentage of cells untreated with 6-hydroxydopamine, which served as the control group and was designated as 100%. The results are expressed as percentage of the control.

2.4. **Preparation of brain MAO.** Rats were decapitated and the brains were quickly taken into a weighted ice-cold sucrose buffer (10 mM Tris–HCl buffer, pH 7.4 containing 0.32 M sucrose), and their weights were measured. The brains were homogenized at 0–4°C in 0.32 M sucrose (one part tissue to 20 parts sucrose) in a Teflon glass homogenizer. The resultant homogenates were used to determine MAO activity. Protein concentration was determined with Bradford reagent at 595 nm, using bovine serum albumin as a standard.

2.5. Determination of MAO activity in vitro. The activity of MAO-A and MAO-B were determined by the adapted method of Tipton & Youdim (Tipton et al. 1983). Briefly, the tested drug was added to a suitable dilution of the enzyme preparation (70 μg protein for MAO-B and 150 μg for MAO-A assay) in 0.05 M phosphate buffer (pH 7.4). The mixture was incubated together with 0.05 μM l-deprenyl, a specific inhibitor of MAO-B (for determination of MAO-A) or 0.05 μM clorgyline a specific inhibitor of MAO-A (for determination of MAO-B). Incubation was carried on 1 hour at 37°C before addition of ^{14}C-5-hydroxytryptamine binoxalate (100 μM) for determination of MAO-A, or ^{14}C-phenylethylamine 100 μM for determination of MAO-B activity, and incubation continued for 30 min or 20 min, respectively. The reaction was stopped with 2 M ice-cold citric acid, and the radioactive metabolites (5-hydroxytryptaldehyde and phenylacetaldehyde) were extracted by addition of 2 ml of ethylacetate/toluene (1:1 vol/vol). The radioactivity contained in the organic phase was determined by liquid-scintillation counting in cpm units.

2.6. Prochelator Activation: For experiments for activating **2**, AChE from Electrophorus electricus (electric eel) (Sigma Company) was used. **2** was incubated with an excess of AChE in 0.1 M sodium phosphate buffer, pH 7.4, containing NaCl 58.5 g/L and Triton X-100 0.05% v/v for 1 h. The metabolite (**M30**) was extracted from the mixture using ethyl acetate and after removal of the solvent and drying under vacuum, was re-dissolved in 5%NH_4Ac (pH = 7). UV/VIS spectra were measured on a Hewlett-Packard 8450A diode array spectrophotometer. The absorption spectra were recorded at room temperature in 1-cm quartz cells, with 5%NH_4Ac (pH = 7) as the reference in the absence and presence of $FeSO_4$, $CuSO_4$ or $ZnCl_2$ (0.1 mM).

3. References

1. Zheng, H., Weiner, L.M., Bar-Am, O., Epsztejn, S., Cabantchik, Z.I., Warshawsky, A., Youdim, M.B., and Fridkin, M. (2005). Design, synthesis, and evaluation of novel bifunctional iron-chelators as potential agents for neuroprotection in Alzheimer's, Parkinson's, and other neurodegenerative diseases. *Bioorg. Med. Chem. 13*, 773-783.