Nitroxide Radical probes as redox-sensitive MRI contrast agents for assessment of radiation-induced damage, neuropathological diseases, and inflammation

Dr. Agnieszka Sulima, Valerie Smith and Jeffrey Lamb

Imaging Probe Development Center, National Heart, Lung, and Blood Institute, National Institutes of Health, 9800 Medical Center Drive, Rockville, MD 20850, ipdc@nhlbi.nih.gov

Background

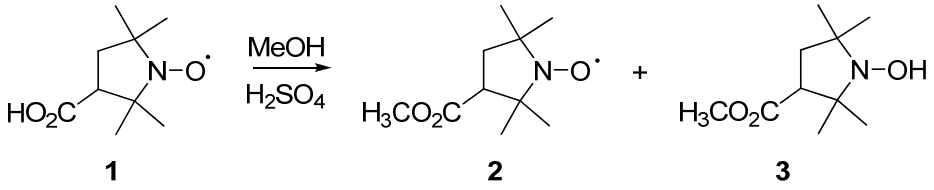
Nitroxides are nontoxic, stable, organic free radicals that have a single unpaired electron and are therefore capable of providing MRI contrast via shortening of longitudinal relaxation time (T_1).[1-4] In several *in vitro* and *in vivo* studies, nitroxides afforded significant protection against the lethal effect of exposure to ionizing radiation.[5, 6] Further studies supported the conclusion that nitroxides and their corresponding one-electron-reduced hydroxylamines are multi-functional protectors against various modes of oxidative stress in both *in vitro* and *in vivo* experimental models.[7] The mechanisms by which they afford protection have been postulated to be mimicry of enzyme activity of superoxide dismutase and peroxidase, radical scavenging, and inhibition of lipid peroxidation.[6, 8-12] Nitroxides and their diamagnetic counterparts, the hydroxylamines, can participate in cellular redox reactions and can be transformed into each other. Among known nitroxides MC-PROXYL[13, 14] and CTMIO[15] (Figure 1) are of particular interest because they are blood-brain permeable and may provide an opportunity to study the redox status of brain tissue, in addition to other tissues throughout the body. Moreover, nitroxide reduction can be studied in other diseases of the brain thought to be mediated in part by oxidative stress, e.g., Parkinson's and Alzheimer's diseases.[16]

Figure 1. Structure of MC-PROXYL (**2**), CTMIO (**8**), and their reduced forms, hydroxylamines **3** and **9**, respectively.

Chemistry

3-Methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine-1-oxyl, (MC-PROXYL), [13, 14] and 5-carboxy-1,1,3,3-tetramethylisoindolin-2-yloxyl, (CTMIO), (**8**)[15] were synthesized according to known procedures with some modifications as noted in the experimental section. The syntheses of MC-PROXYL (**2**) and CTMIO (**8**) are outlined in Schemes 1 and 2, respectively.Commercially available 3-carboxy-2,2,5,5-tetramethylpyrrolidine-1-oxyl, carboxy-PROXYL (**1**) was esterified with methanol in the presence of sulfuric acid (Scheme 1). The resulting mixture of MC-PROXYL), (**2**) and its reduced form, hydroxylamine **3**, was purified by flash chromatography. Both purified MC-PROXYL (**2**) and the hydroxylamine **3** were then characterized by NMR. Since the NMR spectra of the nitroxyl radicals are broad, MC-PROXYL (**2**) was reduced to a diamagnetic *N*-hydroxyl derivative by the addition of 1.5 equivalents of phenylhydrazine in an NMR tube containing the nitroxyl **2** dissolved in deuterochloroform.

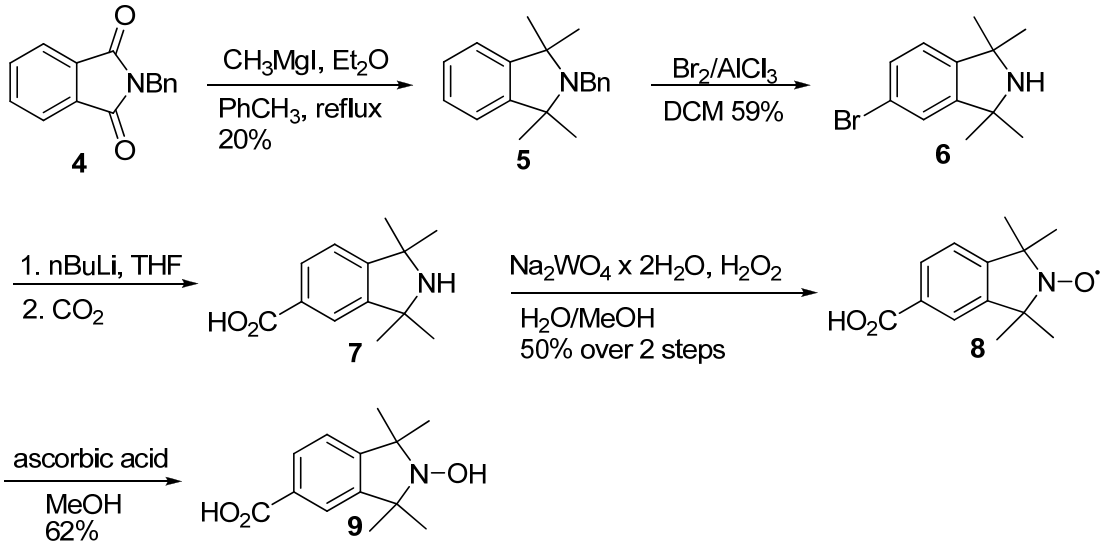
Scheme 1. Synthesis of MC-PROXYL (**2**) and its reduced form, hydroxylamine (**3**).



The synthesis of CTMIO was initiated by a preparation of 2-benzyl-1,1,3,3 tetramethylisoindoline (**5**)[17] in the reaction of *N*-benzylphthalimide (**4**) with excess methyl Grignard reagent (Scheme 2). The synthesis of tetra-alkylisoindoline **5** developed by Griffiths et al.[17] proved to be difficult and involved tedious chromatographic purification. The purification

was performed on an alumina column eluting with petroleum ether and gave the desired compound with low yield. We found that the purification process could be simplified by using silica gel column with hexane as an elution solvent. This provided compound **5** in sufficient purity for the next step. Treatment of **5** with bromine (Br_2) in the presence of anhydrous aluminum chloride ($AlCl_3$) resulted in the oxidative cleavage of the benzyl group and bromination of the isoindoline ring to give 5-bromo-1,1,3,3-tetramethylisoindoline (**6**). Selective monobromination of the isoindoline aromatic ring was highly dependent upon the use of an anhydrous, powdered $AlCl_3$ and an approximately stoichiometric volume of bromine (Br_2). The reaction was also sensitive to solvent and temperature effects with anhydrous dichloromethane (CH_2Cl_2) at 0°C under an atmosphere of argon giving the most consistent results. Lithation with *n*-butyllithium (nBuLi) and subsequent carboxylation of **6** produced 5-carboxy-1,1,3,3-tetramethylisoindoline (**7**). The crude intermediate **7** was then subjected to H_2O_2-tungstate oxidation to generate the target nitroxide **8** (CTMIO). The mass spectrometry (APCI LC-MS) confirmed a mass consistent with the structure of **8**. The purity of product **8** was found to be >97% by analytical HPLC. Finally, the reduction of the nitroxide moiety of **8** to a hydroxylamine **9** was readily achieved with ascorbic acid.

Scheme 2. Synthesis of CTMIO (**8**) and its reduced form, hydroxylamine (**9**).



Experimental

General. Thin-layer chromatography (TLC) analyses were carried out on Analtech (Newark, DE) silica gel GHLF 0.25 mm plates using UV and iodine detection. Melting points were determined in open glass capillaries on an Electrothermal MEL-TEMP 3.0 apparatus. APCI LC-MS mass spectrometry was performed on an Agilent 1200 Series Mass Spectrometer. ^{1}H-NMR and ^{13}C-NMR spectra were recorded on a Varian spectrometer at 400 MHz and 100 MHz respectively. Chemical shifts are reported in ppm using tetramethylsilane (TMS) as the internal standard. Flash chromatography was carried out on a Teledyne Isco Combi*Flash* Companion® instrument with UV detection at 254 nm. Analytical and preparative HPLC analyses were performed on Agilent 1200 Series instruments using an Agilent Zorbax SB-C18 column, 4.6 x 75 mm and an Agilent Prep-C18 column, 30 x 100 mm, respectively. The following method was used in the analytical HPLC analyses: Initial mixture 85% A, 15% B, gradient to 70% A, 30% B over 15 minutes, and then gradient to 5% A, 95% B over 2 minutes, holding for 1 minute at the final concentrations. Solvent A: 0.1% TFA in water; Solvent B: 0.1% TFA in acetonitrile. The following purification method was used in the preparative HPLC runs: Initial mixture 95% A, 5% B, gradient to 65% A, 35% B over 14 minutes, and then to a final mixture of 5% A, 95% B over 2 minutes, holding at the final concentrations for 2 minutes. Solvent A: 0.1% formic acid in water; Solvent B: 0.1% formic acid in acetonitrile. All starting materials and reagents were commercially available from Sigma-Aldrich and were used without further purification unless otherwise noted.

3-Methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine-1-oxyl, (MC-PROXYL), (2)[13, 14] and methyl 1-hydroxy-2,2,5,5-tetramethylpyrrolidine-3-carboxylate (3). To a solution of 3-carboxy-2,2,5,5-tetramethylpyrrolidine-1-oxyl (carboxy-PROXYL) (**1**) (5.9 g, 31.7 mmol) in anhydrous methanol (MeOH, 150 mL) was added dropwise concentrated sulfuric acid (H_2SO_4, 2 mL) and the mixture was stirred for 2 days at room temperature. The solvent was evaporated and the resulting residue was purified by flash chromatography with 15% MeOH in CH_2Cl_2 affording 3.3 g (52%) of free radical **2** as yellow oil and 2.3 g (36%) of hydroxylamine **3** as white hygroscopic crystals, mp 84.6-85°C. MC-PROXYL (**2**) and its reduced form, hydroxylamine **3** were stored under argon at -20°C. The structure of **2** was confirmed by its *in situ* reduction to a

diamagnetic *N*-hydroxyl derivative. To a solution of nitroxyl **2** in $CDCl_3$ in an NMR tube phenylhydrazine (1.5 equivalents) was added and the solution was allowed to stand for several minutes before its spectrum was measured. The NMR spectrum of the *in situ* reduced MC-PROXYL (**2**)[18] was consistent with the spectrum of hydroxylamine **3**: ^{1}H-NMR (400 MHz, $CDCl_3$): δ 3.65 (s, 3H, OCH_3), 2.74 (dd, 1H, *J* = 10.95 and 8.02 Hz, H-3), 2.12 (dd, 1H, *J* = 13.05 and 10.95 Hz, H-4), 1.75 (dd, 1H, *J* = 13.05 and 8.02 Hz, H-4), 1.30 (s, 3H, CH_3), 1.21 (s, 3H, CH_3), 1.13 (s, 3H, CH_3), 1.02 (s, 3H, CH_3); ^{13}C-NMR (100 MHz, $CDCl_3$): δ 173.81, 65.89, 61.93, 51.92, 49.44, 37.96, 27.94, 27.54, 27.07, 20.28. APCI MS *m*/z: 202.27 $(M+H)^+$ (100%).

2-Benzyl-1,1,3,3-tetramethylisoindoline (5).[17] Magnesium turnings (1.18 g, 48 mmol) and anhydrous diethyl ether (8 mL) were placed in a 0.5-litre three-necked flask equipped with a magnetic stirrer and an efficient reflux condenser under an atmosphere of argon. A solution of methyl iodide (3.2 mL, 51 mmol) in anhydrous diethyl ether (40 mL) was added dropwise from a dropping funnel at a rate to maintain the solvent at reflux. When the addition of methyl iodide solution was completed, the mixture was refluxed for 30 minutes and a solution of *N*-benzylphthalimide (1.74g, 7.32 mmol) (**4**) in toluene (140 mL) was added dropwise over 20-25 minutes. Once the addition was complete, solvent was distilled slowly from the mixture until the temperature reached 108-110°C. After being maintained at this temperature for 5 hours, the mixture was concentrated to approximately 50 mL by further solvent distillation. It was cooled to room temperature and diluted with petroleum ether (80 mL). The resulting slurry was stirred in air for 2 hours (during which time the solution turned purple), filtered through a pad of Celite and washed with petroleum ether (3 x 100 mL). The solvent was evaporated under reduced pressure and the residue was purified by flash chromatography on silica gel eluting with hexanes. Evaporation of the pale yellow solution gave product **5** (0.39 g, 20%). An analytical sample was further purified by crystallization form ethanol as white crystals, mp 57.5-59.4°C. ^{1}H-NMR (400 MHz, $CDCl_3$): δ 7.47-7.12 (m, 9H), 3.99 (s, 2H), 1.29 (s, 12H). ^{13}C-NMR (100 MHz, $CDCl_3$): δ 148.07, 143.67, 128.53, 128.12, 126.97, 126.61, 121.52, 65.39, 46.44, 28.61. APCI MS *m*/z: 266.4 $(M+H)^+$ (100%).

5-bromo-1,1,3,3-tetramethylisoindoline (6).[15] 2-Benzyl-1,1,3,3-tetramethylisoindoline (**5**) (4.8 g, 18 mmol) was dissolved in anhydrous CH_2Cl_2 (60 mL) under an atmosphere of argon and

cooled to 0°C. A solution of Br_2 (2.03 mL, 39.6 mmol) in anhydrous CH_2Cl_2 (30 mL) was added with stirring followed immediately by the addition of powdered anhydrous $AlCl_3$ (8.4 g, 63 mmol). The reaction mixture was stirred at 0°C for 1.5 hours and then poured onto ice (150 mL). After 20 minutes, the solution was basified with 10M sodium hydroxide (NaOH) and extracted with CH_2Cl_2 (3 x 60 mL). The combined organic phases were washed with brine and the solvent removed under reduced pressure. The resulting golden oil was taken up in MeOH (25 mL) and sodium bicarbonate ($NaHCO_3$, 200 mg) added followed by 30% hydrogen peroxide (H_2O_2, 2 mL). The mixture was stirred for 15 minutes, then treated with 2M H_2SO_4 (70 mL), and washed with CH_2Cl_2 (2 x 50 mL) to remove benzaldehyde. The combined organic phases were further extracted with 2M H_2SO_4 (2 x 30 mL) and the combined acidic phases washed with CH_2Cl_2. The acidic phase was cooled in an ice-water bath, basified with 10M NaOH and extracted with CH_2Cl_2 (3 x 60 mL). The combined organic phases were washed with brine, dried over anhydrous magnesium sulfate ($MgSO_4$) and evaporated under reduced pressure to give a golden oil which rapidly crystallized. The resulting 5-bromo-1,1,3,3-tetramethylisoindoline (**6**) (2.68 g, 59%) was of sufficient purity, according to TLC and ^{1}H NMR, for subsequent use without further purification. ^{1}H-NMR (400 MHz, $CDCl_3$): δ 7.35 (dd, 1H, J = 8.12 and 1.76 Hz), 7.23 (d, 1H, J = 1.76 Hz), 6.98 (d, 1H, J = 8.12 Hz), 1.82 (b, 1H, N*H*), 1.44 (s, 6H), 1.43 (s, 6H). APCI MS *m*/z: 254/256.3 $(M+H)^+$ (100%).

5-Carboxy-1,1,3,3-tetramethylisoindolin-2-yloxyl (8), (CTMIO).[15] *n*-Butyllithium (1.6 M in hexane, 9.88 mL, 15.8 mmol) was added to a solution of **6** (1.68 g, 7.18 mmol) in anhydrous tetrahydrofuran (THF, 20 mL) at -78°C under an atmosphere of argon. After 10 minutes the reaction mixture was poured onto a slurry of powdered dry ice in anhydrous THF (80 mL total volume). It was stirred until it reached room temperature. The solvent and other volatiles were removed under reduced pressure. The residue was taken up in 2N hydrochloric acid (HCl, 30 mL) and extracted with diethyl ether (3 x 20 mL) to remove pentanoic acid and neutral organics. The aqueous phase was then neutralized with a saturated solution of sodium carbonate (Na_2CO_3) and extracted again with diethyl ether to remove basic species. The aqueous phase was then lyophilized yielding a white solid residue containing inorganic salts and product **7**. The crude solid was dissolved in water/methanol (1:1) (25 mL), and $NaHCO_3$ (482 mg, 5.74 mmol), sodium tungstate (Na_2WO_4 x $2H_2O$, 213 mg, 0.65 mmol) and 30% H_2O_2 (2 mL) added. After

stirring at room temperature for 24 h, another portion of 30% H_2O_2 (1 mL) was added. After an additional 48 hours of stirring, the reaction mixture was basified with 2M NaOH and extracted with diethyl ether (3 x 30 mL) to remove basic species. The aqueous phase was then acidified with 5N HCl and extracted with diethyl ether (3 x 30 mL). The combined organic phases were washed with brine, dried over $MgSO_4$, and the solvent removed under reduced pressure. The crude product **8** was crystallized from acetonitrile (CH_3CN) yielding 0.85 g (50%) of 8 as a yellow powder, mp 214.5-216.1°C (lit. 214-218°C). The purity of **8** was found to be >97% by analytical HPLC following the conditions outlined in the General Experimental section, retention time: 11.69 minutes. APCI MS *m*/z: 236.4 $(M+2H)^+$ (32%), 220 (100%), 204 (37%), 189(10%).

2-Hydroxy-1,1,3,3-tetramethylisoindoline-5-carboxylic acid (9). A solution of **8** (0.15 g, 0.64 mmol) in anhydrous MeOH (5 mL) was deoxygenated by bubbling with argon for 15 minutes. Ascorbic acid (0.39 g, 2.24 mmol) was then added and the reaction mixture was stirred for 24 h at room temperature. The solvent was removed under reduced pressure and the resulting residue was purified by preparative HPLC following the conditions outlined in the General Experimental section. Compound **9** was obtained as a white solid (0.93 g, 62%), mp 219.5-222°C. ^{1}H-NMR (400 MHz, DMSO-d_6): δ 7.83 (d, 1H, J = 7.82 Hz), 7.74 (s, 1H), 7.65 (b, 1H), 7.30 (d, 1H, J = 7.82 Hz), 1.32 (s, 6H), 1.31 (s, 6H). ^{13}C-NMR (100 MHz, DMSO-d_6): δ 168.17, 150.93, 146.44, 131.18, 129.22, 123.35, 122.48, 66.68, 66.52, 27.02, 26.88. The purity of **9** was found to be >89% by analytical HPLC following the conditions outlined in General Experimental section, retention time: 3.80 minutes; APCI MS *m*/z: 235.5 $(M+H)^+$ (100%), 219.9 (31%), 203.9 (32%), 190 (8%).

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