

Synthesis of CLaNP-5 for use as an NMR Probe

Kyle Barbacow and Olga Vasalatiy

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

The unpaired electrons of paramagnetic lanthanide ions can influence the NMR spectra of a protein and its complexes by causing tunable shifts or broadening of resonances within the spectra. A series of lanthanide ions can be immobilized on the conformationally restricted caged lanthanide NMR Probe 5 (CLaNP-5, **8**) and then bound to an engineered protein surface.¹ Subsequently, through various paramagnetic NMR experiments, specific calculations can be interpreted and used to refine the structure of a protein and the associated complexes formed within.

Chemistry:

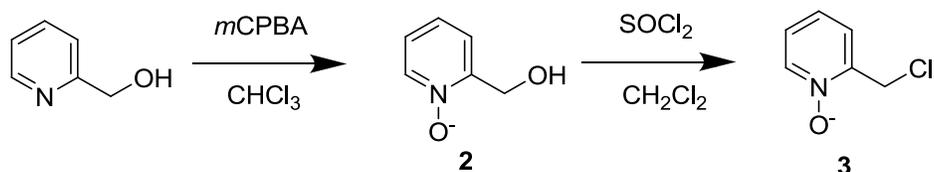
1,4,7,10-Tetraazacyclododecane-1,7-[di-(*N*-oxido-pyridine-2-yl)methyl]-4,10-bis(2-(acetylamino)ethylmethanesulfonylthioate) (CLaNP-5, **8**) was obtained as outlined in **Schemes 1** and **2**.

Synthesis of 2-(chloromethyl)pyridine-*N*-oxide (**3**) is shown in **Scheme 1** where 2-(hydroxymethyl)-pyridine (**1**) was first oxidized using *meta*-chloroperoxybenzoic acid (*m*-CPBA) to provide the pyridine-*N*-oxide analog **2**. Pyridine-*N*-oxide analog **2** was transformed into the corresponding chloride **3** using thionyl chloride.¹

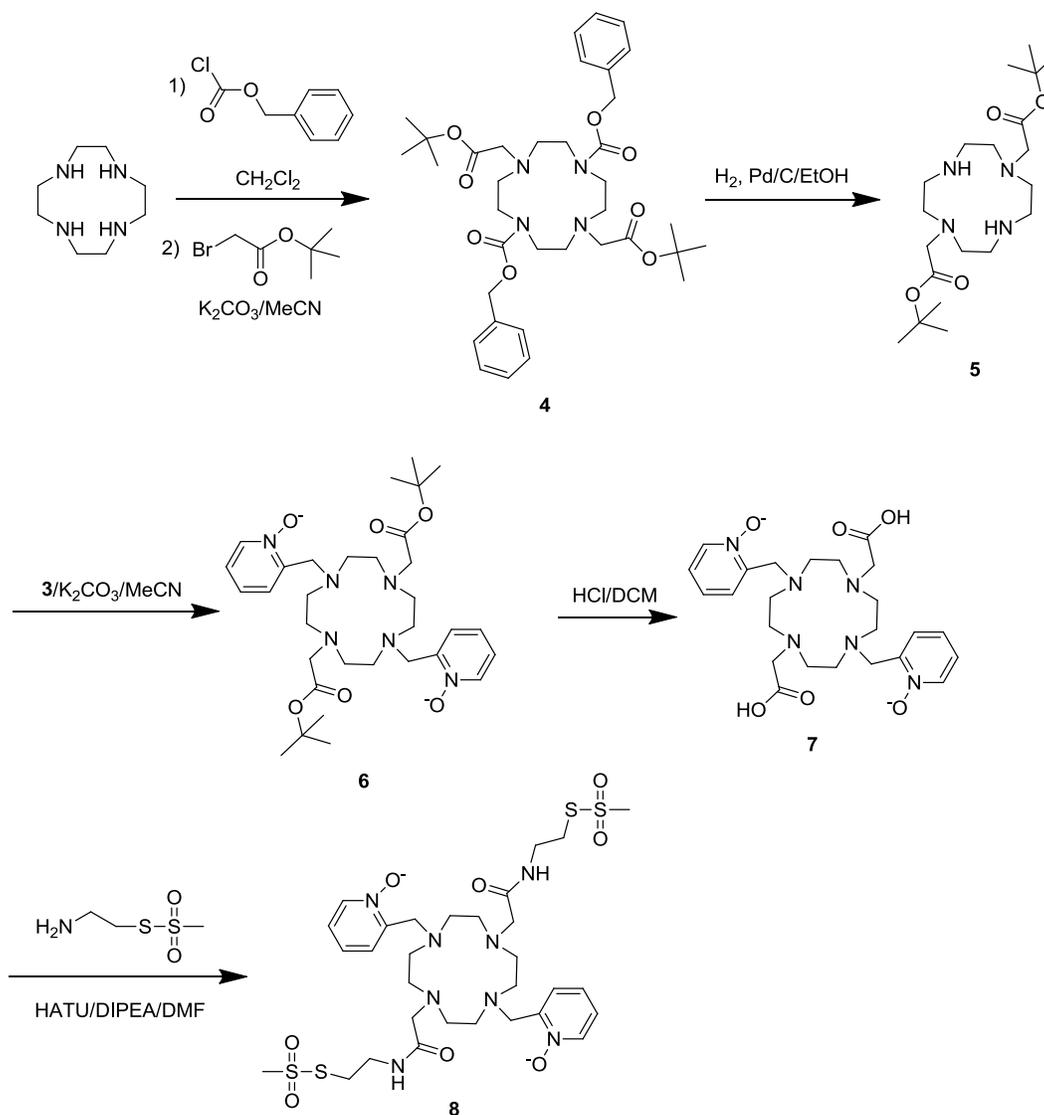
Alkylation of tetraazacyclododecane with benzyl chloroformate to obtain the bis-protected cyclen derivative was accomplished according to a published protocol², followed by subsequent alkylation with *tert*-butyl bromoacetate in the presence of base to afford the intermediate **4**. Facile removal of the benzyloxycarbonyl group by catalytic hydrogenation yielded cyclen

derivative **5**. Cyclen derivative **5** was bialkylated with chloride **3** to give the diacid **7** after deprotection. 2-(Aminoethyl)methanesulfonate hydrobromide (MTSEA) was then coupled to diacid **7** in presence of 2-(1H-7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyl uronium hexafluorophosphate methanaminium (HATU) and diisopropylethylamine (DIPEA) to provide CLaNP-5 (**8**).

Scheme 1. Synthesis of 2-(chloromethyl)pyridine-*N*-oxide



Scheme 2. Synthesis of CLaNP-5



Experimental

General: All organic precursors and solvents were obtained from commercial sources and used as received. 2-(Aminoethyl)methanethiosulfonate hydrobromide was obtained from Toronto Research Chemicals (North York, Canada). Hydrogenations were performed on a Parr hydrogenation apparatus. ^1H and ^{13}C NMR spectra were acquired using a Varian spectrometer operating at 400 and 100 MHz, respectively. Chemical shifts are reported in parts per million (δ)

and referenced to tetramethylsilane (TMS). Thin-Layer Chromatography (TLC) analyses were performed with Analtech (Newark, DE) silica gel GHLF 0.25mm plates using UV and iodine detection. Flash chromatography was performed on an Analogix IntelliFlash 280 instrument with UV detection at 254 nm. Analytical HPLC analyses were performed on Agilent 1200 series instruments equipped with multi wavelength detectors using an Agilent Zorbax SB C18 (4.6 x 50mm, 3.5µm) at 1.00 mL/min flow rate. Solvent A was 0.05% trifluoroacetic acid in water, Solvent B was 0.05% trifluoroacetic acid in acetonitrile, and a gradient of 5-95% over 7 min was used. APCI mass spectrometry (APCI-MS) was performed on LC/MSD TrapXCI Agilent Technologies. Melting points were obtained using an Electrothermal MEL-TEMP 3.0 scientific melting apparatus (Barnstead International, Dubuque, IA).

2-(Hydroxymethyl)pyridine-*N*-oxide (2). 2-(hydroxymethyl) pyridine (60.0 g, 550 mmol) was dissolved in chloroform (900 mL) at 0 °C. To this cold mixture *m*-CPBA (147.9 g, 77%, 660 mmol) was added and stirred at 0 °C for 30 min. After 30 min, the ice bath was removed and stirring was continued at room temperature for 15 h. Paraformaldehyde (12.0 g, 400 mmol) was added and the mixture was stirred for 2 h to quench the residual *m*-CPBA,. Ammonia was bubbled through the suspension for 1.5 h. The resulting white slurry's volume was reduced under vacuum and subsequently washed with dichloromethane (1.4 L) and continuously extracted for 48 h. The solvents were removed under reduced pressure, and the mixture was purified using silica gel chromatography (methanol/dichloromethane/acetic acid, 0.4:9:0.1 to 1.4:8.5:0.1). Removal of solvents under reduced pressure afforded white crystal needles (38.44 g, 56%). ¹H NMR (400 MHz CDCl₃): δ = 4.78 (2H, s, Ar-CH₂), 7.21 (1H, t, Ar), 7.30 (1H, t, Ar), 7.34 (1H, t, Ar), 8.21 (1H, d, Ar). ¹³C NMR (100.56 MHz, CHCl₃): δ = 61.5, 124.8, 125.0, 127.6, 139.8,153.8. *m/z* (APCI MS) 126.6 ([M+H]⁺).

2-(Chloromethyl)pyridine-*N*-oxide (3). 2-(hydroxymethyl)pyridine-*N*-oxide (38.44 g, 307.2 mmol) was dissolved in anhydrous dichloromethane (1.24 L) at 0 °C and stirred for 15 minutes. Thionyl chloride (39.68 g, 333.5 mmol) was then added dropwise with a syringe over a period of 1 h. The reaction was warmed to room temperature and refluxed for 1 h. Ethanol (12 mL) was added to quench the residual thionyl chloride. The solvents were removed under reduced pressure and the residue was recrystallized from acetone giving 38.0 g (86%) of compound **3** as yellow needles. MP 108-110 °C. ¹H NMR (400 MHz CDCl₃): δ = 5.03 (2H, s, Ar-CH₂), 7.81

(1H, t, Ar), 8.08 (2H, m, Ar), 9.05 (1H, d, Ar). ¹³C NMR (100.56 MHz, CHCl₃): δ = 38.6, 126.8, 127.6, 138.1, 140.6, 148.8. *m/z* (APCI MS) 144.2 ([M]⁺).

1,7-Bis(benzyloxycarbonyl)-4,10-bis(*tert*-butoxycarbonyl methyl)-1,4,7,10-

tetraazacyclododecane (4). Potassium carbonate (39.97 g, 289.2 mmol) was added to a solution of bis-protected cyclen (42.37 g, 96.18 mmol) in acetonitrile (20 mL) and the suspension was heated at 70 °C for 10 minutes under an argon atmosphere. *tert*-Butyl bromoacetate (39.97 g, 204.92 mmol) was then added dropwise. The reaction mixture was heated for 48 hours at 65-70 °C, filtered and the solvent was removed under reduced pressure. The resulting residue was adsorbed on silica gel and purified by flash chromatography (EtOAc/Hexanes/TEA 2.8:7:0.2). Solvents were removed to afford a colorless oil (44.50 g, 69.2%). ¹H NMR (400 MHz, CDCl₃): δ = 1.43 (18H, s, CH₃), 2.88 (8H, br, CH₂), 3.21-3.42 (12H, m, ring CH₂, CH₂COO), 5.12 (4H, CH₂Ph), 7.34 (10H, m, Ph). ¹³C NMR (100.56 MHz, CHCl₃): δ = 28.24, 46.96-47.32(br), 54.66-54.31 (br), 56.26, 67.26, 81.19, 128.12, 128.16, 128.70, 137.10, 156.71, 170.79. *m/z* (APCI MS) 669.6 ([M+H]⁺).

1,7-(*Tert*-butoxycarbonyl methyl)-1,4,7,10-Tetraazacyclododecane (5). 10% Palladium on carbon (0.2 g) was added to a solution of compound 7 (44.50 g, 66.53 mmol) in ethanol (30 mL). The reaction mixture was placed in a hydrogenation vessel for 12 h with the H₂ pressure set to 50 psi. The catalyst was filtered through Celite[®]545 (Aldrich) and the solvents were removed under reduced pressure to afford a colorless oil (25.81 g, 97 % yield). ¹H NMR (400 MHz, CHCl₃): δ = 1.46 (18H, s, CH₃), 2.63 (8H, t, J= 5 Hz, CH₂ ring), 2.82 (8H, s br, CH₂ ring), 3.32 (4H, s, CH₂COO). ¹³C NMR (100.53 MHz, CHCl₃) δ = 28.42, 46.03, 52.27, 57.83, 81.14, 171.89. *m/z* (APCI MS) 401.4 ([M+H]⁺).

1,4,7,10-Tetraazacyclododecane-1,7-[di(*N*-oxido-pyridine-2-yl)methyl]-4,10-bis(di-*tert*-butyl ester (6). Potassium carbonate (18.70 g, 135.31 mmol) was added to a solution of compound 8 (25.81 g, 64.43 mmol) in acetonitrile (200 mL) and the suspension was heated at 70°C for 10 minutes. 2-(Chloromethyl)pyridine-*N*-oxide (19.43 g, 135.31 mmol) was slowly added and the reaction mixture was stirred at 70°C for 8 h. Water (50 mL) was added to the reaction mixture which was extracted with dichloromethane (3x200 mL), and combined organic

phase was dried over MgSO₄. The dried organic phase was concentrated under reduced pressure, and purified by flash chromatography (dichloromethane:methanol =5:1) (35.0 g, 88.6%). ¹H NMR (400 MHz CDCl₃): δ = 1.39 (18H, s, C(CH₃)), 2.90 (16H, m, CH₂ ring), 3.44 (4H, s, CH₂COOtBu), 3.85 (4H, s, CH₂ Ar), 7.20 (2H, m, Ar), 7.40 (2H, m, Ar), 7.70 (2H, d, Ar), 8.38 (2H, d, Ar). ¹³C NMR (100.53 MHz, CD₃Cl₃) δ = 28.7, 52.1, 53.8, 54.0, 56.1, 78.1, 123.1, 126.2, 138.0, 140.1, 148.1, 169.1 *m/z* (APCI MS) 615.6 *m/z* ([M+H]⁺).

1,4,7,10-Tetraazacyclododecane-1,7-[di-(*N*-oxido-pyridine-2-yl)methyl]-4,10-diacetic acid (7). Compound **6** (35.0 g, 56.9 mmol) was dissolved in 4 M HCl in dichloromethane and stirred at room temperature for 3 hours until an off white precipitate formed. The reaction mixture was filtered and dried to yield an off-white solid (25.0 g, 87.4%). ¹H NMR (400 MHz, D₂O): δ = 2.65 (16H, s, CH₂ ring), 3.65 (4H, s, CH₂COOH), 3.95 (4H, s, CH₂ Ar), 7.50 (2H, m, Ar), 7.69 (4H, m, Ar), 8.33 (2H, d, Ar). ¹³C NMR (100.53 MHz, CH₃OH) δ = 49.80, 49.85, 51.89, 59.28, 126.01, 129.15, 131.05, 140.12, 148.3. 179.33 *m/z* (APCI MS) 503.4 ([M+H]⁺).

1,4,7,10-Tetraazacyclododecane-1,7-[di-(*N*-oxido-pyridine-2-yl)methyl]-4,10-bis(2-(acetylamino)ethylmethanesulfonylthioate) (8). Compound **7** (2 g, 4 mmol), 2-(aminoethyl)methanethiosulfonate (1.6 g, 8.4 mmol) and DIPEA (2 mL) were dissolved in DMF(10 mL). The reaction mixture was cooled in an ice bath for 5 minutes, followed by the addition of HATU (3.2 g, 8.4 mmol). The reaction was warmed and stirred overnight at room temperature. One fourth of the reaction mixture was injected into a pre-equilibrated (water/0.1%TFA) RediSep R_f column (240 g C18 RP) and purified by flash chromatography (water (0.1%TFA):acetonitrile (0.1%TFA) from 100:0 to 91:9 over 60 min). The fractions containing the title compound were analyzed by analytical HPLC (R_t = 2.7 min), combined, and solvents were removed under reduced pressure. The flash chromatography purification of the combined fractions was repeated a second time to obtain 98% purity as assessed by analytical HPLC (1.4 g, 45% yield). ¹H NMR (400 MHz, D₂O): δ = 3.20-3.62 (36H, m, CH₂ ring, CH₂-SSO₂CH₃,), 4.50 (4H, s, CH₂Ar), 7.65 (2H, d, Ar), 7.77 (2H, d, Ar), 7.98 (2H, d, Ar), 8.42 (2H, d, Ar). *m/z* (APCI MS): 777.3 ([M]⁺).

References:

1. Keizers PH, Saragliadis A, Hiruma Y, Overhand M and Ubbink M. Design, synthesis, and evaluation of a lanthanide chelating protein probe: CLaNP-5 yields predictable paramagnetic effects independent of environment. *J Am Chem Soc.* 2008 Nov 5;130(44): 14802-12. PMID 18826316.
2. De León-Rodríguez LM, Kovacs Z, Esqueda-Oliva AC and Miranda-Olvera AD. Highly regioselective N-trans symmetrical diprotection of cyclen. *Tetrahedron Letters.* 2006 47(39): 6937-6940.
3. Bremberg U, Rahm F and Moberg C. Palladium-catalyzed allylic alkylation using pyridino-oxazolines and quinolino-oxazolines as ligands--influence of steric factors. *Tetrahedron: Asymmetry.* 1998 9(19): 3437-3443.