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SYNTHESIS AND RELAXIVITY MEASUREMENT OF CYCLEN BASED MAGNETIC RESONANCE IMAGING (MRI) CONTRAST AGENT**

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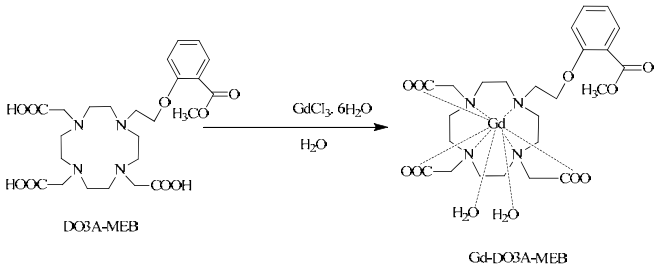
1, 4, 7, 10-teraazacyclododecane (cyclen) is a key macrocycle used for the synthesis of Magnetic Resonance Imaging (MRI) contrast agents. Substituting the hydrogen atoms (attached to the nitrogen atoms in the ring) of cyclen with suitable groups produce stable ligands. These ligands on complexation with Ln^{3+} ions lead to the formation of useful chelates. Cyclen is widely synthesized via Richman and Atkins method. Cyclen based ligand 1,4,7-*tris*(carboxymethyl)-10-(methyl,2-ethoxybenzoate)-1,4,7,10-tetraazacyclododecane (DO3A-MEB) was synthesized in this work. It was subsequently coupled with Gd^{3+} ion to afford its stable complex. Relaxivity of the complex was measured by inversion recovery (IR) method and it was compared with that of the commonly used MRI contrast agent Gd-DTPA. Longitudinal relaxivity measurements indicated 128 % enhancement compared to Gd-DTPA.



DO3A-MEB Gd-DO3A-MEB

INTRODUCTION

1, 4, 7, 10-Teraazacyclododecane (cyclen) is a macrocycle, the aza analogue of the crown ether. The most important application of cyclen is the development of Magnetic Resonance Imaging (MRI) contrast agents by its derivatization followed by complexation with Ln^{3+} ions. Diagnostic and therapeutic pharmaceutical agents are developed by the proper substitution of its ring nitrogen atoms. In stable cyclen based-macrocyclic chelates three or four of the ring nitrogen atoms carry a pendant, ionizable metal coordinating group such as phosphonic or carboxylic acid group. As lanthanide ions are in +3 state, the complexation of cyclen-based ligands containing three acid groups with suitable lanthanide ions results in the formation of neutral chelates. This effect is of specific importance because various side effects of contrast agents depend on the hypertonicity. Neutral complexes of lanthanide ions also show small contribution to the overall osmolality of the composition. Such neutral complexes (with low osmolality) decrease the pain and tissue sloughing during the injection. Therefore, cyclen is considered as a crucial intermediate for the synthesis of suitable macrocyclic chelating agents.[1-3] For imaging purposes, various cyclen-based chelates are used as contrast agents. These chelates serve as carriers for the paramagnetic metal ions (gadolinium and dysprosium) and thus ensure their appropriate biodistribution and elimination.[4]

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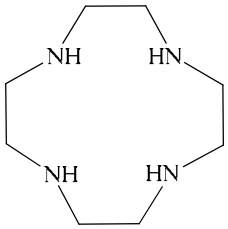


Fig. 1 – Structure of 1,4,7,10-tetraazacyclododecane (Cyclen) .

SYNTHESIS

Keeping in view the immense importance of cyclen as building block for the synthesis of novel MRI contrast agents, a number of synthetic routes have been reported in the literature among which some of the key synthetic methods are described here.

a) Richman and Atkins Procedure

This is one of the most commonly used methods for the synthesis of cyclen, consisting of multistep protection-deprotection strategy.[5-7] The key step of this method involves reaction of a preformed salt of a tritosylamide with sulfonate esters as leaving group in N,N-Dimethylformamide (DMF). Final separation of cyclen needs severe conditions to remove the tosyl protecting groups. For this purpose, 97% H_2SO_4 is used. This is a useful method provided that care is taken in the use of pure and dry starting materials. It has been reported to produce cyclen with a high yield (more than 60 %).

b) Weisman and Reed Synthesis

This is an easy method to implement in the laboratory for the synthesis of cyclen.[8] However, this procedure requires expensive starting materials and reactants. This method utilizes two steps synthesis of cyclen from triethylenetetraamine and dithiooxamide. An important step is the double reductive ring expansion (in refluxing toluene for 15 h producing cyclene of more than 90% purity) of tricyclic bis-amidine with Diisobutylaluminium hydride (DIBALH) to produce cyclen. This reduction step is carried out in refluxing toluene for 15 h producing cyclene of more than 90% purity. The overall yield of cyclen has been reported to be about 57%. However, a drawback of this method is the formation of byproduct "ethanethiol" which needs to be flushed from the reaction mixture.

c) Cyclen synthesis via cyclization of bis-imidazoline with 1,2-dibromoethane

This novel synthetic route was suggested by Athey and Kiefer. [2] This is a three-step procedure with an overall yield of about 65%. Condensation of triethylene tetraamine (TETA) with N,N-dimethylformamide dimethyl acetal produces bis-imidazoline with a yield of more than 90%. Bis-imidazoline then on cyclization with 1,2-dibromoethane gives the twelve-membered, imidazolinium, cyclized intermediate bromide salt with a yield of about 70%. This intermediate bromide salt hydrolyses only at high pH (>12). Therefore, it is treated with hot aqueous caustic to produce cyclen with a yield of more than 80%.

RESULTS AND DISCUSION

For cyclen synthesis, all the above procedures were carefully attempted in the laboratory. However, Richman and Atkins method was adopted due to its cheaper starting materials and high product yield.[5] In the next step cyclen on reaction with *t*-butyl bromoacetate furnished 1,4,7-tris-(*tert*-butoxycarbonylmethyl)-1,4,7,10-tetraazacyclododecane, hydrogen bromide (DO3A-*tert*-butyl.HBr). This compound is commercially available. However, commercially available form is not only expensive but also contains impurities of both the di-alkylated and tetra-alkylated cyclen. Therefore, to obtain DO3A-*tert*-butyl.HBr of high purity, it was prepared in the laboratory. This protected form of DO3A is a ligand by itself and also a key intermediate in the synthesis of useful MRI contrast agents. It is derivatized at the free macrocyclic nitrogen for the preparation of a variety of bifunctional ligands, whose metal complexes are useful for imaging and therapy. DO3A-*tert-butyl*.HBr was prepared in high yield. The reaction is easy to handle and no specific reagents or harsh conditions are needed. Purification was done on column chromatography. Chloroform was found to be the best solvent for this experiment. The use of chloroform is preferred because polar, aprotic solvents (such as DMF) and polar, protic solvents (such as methanol) leads to significant increase in tetra-(*tert*-butoxycarbonylmethyl)-1,4,7,10- tetraazacyclododecane as a byproduct by promoting the proton transfer. Therefore, use of solvents like DMF and methanol for the synthesis of DO3A-*tert*-butyl.HBr

results in its low yield (40-60%). Among the bases tested, triethylamine produced the best yield. Highest yield (77%) of compound was obtained when 3.5 equivalent of *t*-butyl bromoacetate were added. The effects of both temperature and reaction time on the yield of compound were monitored. A temperature range of 20-35^{o}C was found to be suitable. A reaction time of 16-20 h gave the best yield. High temperature resulted in the formation of tetra-(*tert*-butoxycarbonyl-methyl)-1,4,7,10-tetraazacyclododecane probably due to the breaking of H-bond between the nitrogen atoms in the cyclen ring. Furthermore, no improvement in the product yield was found by extending the reaction time.[4, 9, 10] In the next step, methyl salicylate or wintergreen oil was synthesized by the esterification of Salicyclic acid with methanol in the presence of Thionyl Chloride. This step was carefully performed as thionyl chloride is very sensitive towards moisture. Therefore, both glassware and solvent were absolutely dry.[11-13] Methyl Salicylate on treatment with 1,2-dibromoethane furnished 2-(2-bromoeth-oxy)-benzoic acid methyl ester.[14,15] DO3A-*tert-butyl*.HBr on treatment with 2-(2-bromoethoxy)-benzoic acid methyl ester produced 1, 4,7-tris(*tert*-butoxycarbonylmethyl)-10 -(methyl, 2-ethoxy-benzoate)1,4,7,10- tetrazacyclododecane (DO3A-*tert*-butyl-MEB). [16, 17] In the final step *tert*-butyl protective group were removed by the treatment of DO3A-*tert*-butyl-MEB with trifluoroacetic acid (TFA) in dichloromethane at room temperature.[18,19] Thus novel ligand 1,4,7-*tris*(carboxymethyl)-10-(methyl,2-ethoxybenzoate)-1,4,7,10-tetraazacyclo-dodecane (DO3A-MEB) was obtained. Finally, gadolinium complex of DO3A-MEB was obtained by treating it with $GdCl_3.6H_2O$ in water.[20]

Relaxivity test of Gd- DO3A-MEB

The effectiveness of an MRI contrast agent is usually measured in terms of its relaxivity, defined as the increase in water proton relaxation rate per unit concentration of contrast agent. Contrast agents with high relaxivity cause greater contrast at equivalent doses than contrast agents with lower relaxivity.[21] Ligand DO3A-MEB has a large molecular weight as it was obtained by coupling DO3A-*tert*-butyl.HBr with 2-(2-bromoethonoxy)-benzoic acid methyl ester. Therefore, it is expected that Gd-DO3A-MEB will exhibit high relaxivity. For the measurement of longitudinal relaxation time T_1, solutions of certain concentration of complexes Gd-DTPA and Gd- DO3A-MEB in distilled water were separately prepared. A 2 mmol solution of Gd-DTPA (5.64 mg in 5 mL of solution) was prepared. Similarly 2 mmol solution of the complex Gd-DO3A-MEB (7.1 mg in 5 mL solution) was prepared in a separate flask. pH of both the solutions was adjusted to 7. Longitudinal relaxation time $(T1)_d$ of pure water and longitudinal relaxation time $(T_1)_{obs}$ of water in the presence of the paramagnetic complex were measured with the method of inversion recovery (IR) on a Bruker BIOSPECT 47/30 magnetic resonance imaging (4.7 Tesla) spectrometer.[22] Spin-lattice relaxation rate $(1/T_1)_d$ of pure water and observed relaxation rate $(1/T_1)_{obs}$ of water in the presence the complex Gd-DO3A-MEB were measured under the same conditions and then equation (1) was used to calculate relaxivity (R_1) of the complex.[23, 24]

$$R_1[M] = (1/T_1)_{obsd} — (1/T_1)_d \quad (1)$$

Where [M] indicates concentration of Gd^{3+}

Table 1

Relaxivity of gadolinium complexes

Gd^{3+} Complex	Molecular weight (ligand)[g/mol]	[M] [mmol l^{-1}]	$T_{1(obsd)}$ [s]	R_1/Gd [L^{-1}·mmol·s] $^{-1}$	Enhancement	(%)
Gd-DO3A-MEB	524	1.31	0.112	6.53	128	
Gd-DTPA	393	1.241	0.1498	5.09	100	

$T_{1(d)}$= 2.798 s; temperature: 25 ˚C; frequency: 200 MHz

$T_{1(d)}$ represents Longitudinal relaxation time of pure water, $T_{1(obs)}$ represents observed Longitudinal relaxation time of water in the presence of paramagnetic complex, [M] is the concentration of Gd^{3+} ion, R_1/Gd^{3+} indicates the ratio of the relaxation rate to the concentration of the Gd^{3+} ion.

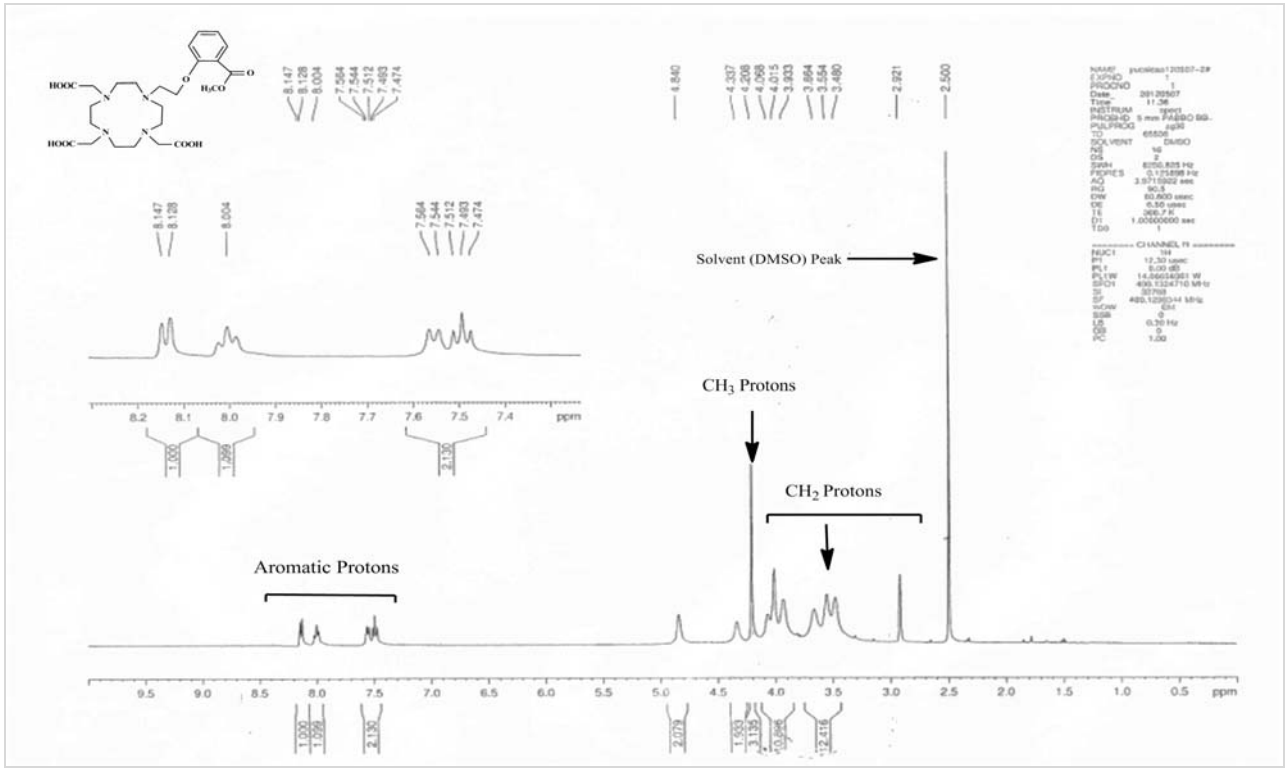


Fig. 2 – ^{1}HNMR spectrum of ligand DO3A-MEB.

The results of the relaxation performance test show that compared to the current widely used clinical MRI contrast agent, Gd-DTPA, the relaxation performance of the complex synthesized in this work has been improved. Enhanced longitudinal relaxivity was observed due to large molecular weight of the complex Gd- DO3A-MEB. Increase in size of molecules cause them to rotate slowly in aqueous solution and therefore lead to their prolonged rotational correlation time. The inner sphere proton relaxivity is linearly proportional to the hydration number (q) of Gd^{3+}. Gd- DO3A-MEB possesses greater hydration number (q = 2) than Gd-DTPA (q = 1). All these factors together contribute to enhanced longitudinal relaxivity (128 % enhancement) of the complex Gd-DO3A-MEB.

EXPERIMENTAL

All reagents were of analytical grade and were distilled, crystallized or used without further purification, as appropriate. Melting points of the compounds were determined in open capillaries on Gallenkamp melting point apparatus and are uncorrected. The IR spectra were recorded on Bruker FT-EQUINOX55 IR spectrophotometer as KBr discs. ^{1}HNMR were recorded on Bruker AV 400 (400 MHz) using TMS as internal standard. Silica gel (particle size 200-300 mesh) used for column chromatography was purchased from Qingdao Marine Chemical Factory. Gd-DTPA was purchased from Shandong Hongfuda Pharm chem Company Limited Shandong. $GdCl_3.6H_2O$ was purchased from Hangzhou Ocean Chemical Co., Ltd. Zhejiang.

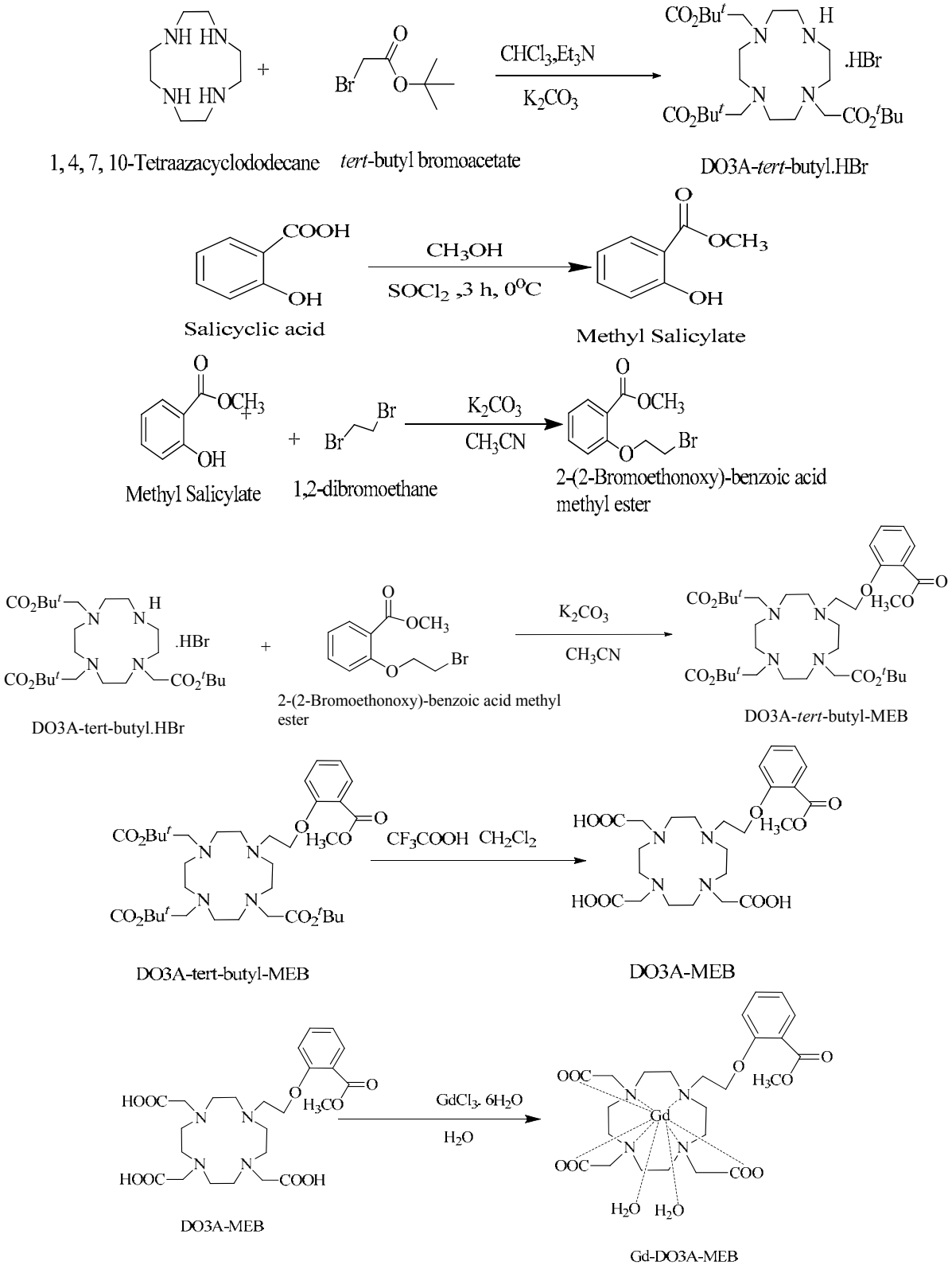
Synthesis of 1, 4, 7-tris-(*tert*-butoxycarbonylmethyl)-1, 4, 7, 10-tetraazacyclododecane, hydrogen bromide (DO3A-*tert*-butyl.HBr)

3.3 equivalent of *tert*-butyl bromoacetate (773 mg, 7.6 mmol) dissolved in 10 mL anhydrous chloroform was added dropwise to a mixture of cyclen (400 mg, 2.32 mmol) and 10 equivalent of triethylamine (2.3 g, 23.2 mmol) dissolved in 40 mL anhydrous chloroform under nitrogen atmosphere at 0^{o}C in about 0.5 h. The reaction mixture was stirred for another 2 h and then 0.5 equivalent of anhydrous K_2CO_3 (160 mg, 1.16 mmol) was added. After further 14 h of stirring, the resulting solution was washed by water (3×40 mL). Then anhydrous Na_2SO_4 was used to dry the organic phase. The solvent was removed via rotary evaporator to obtain transparent oil. The crude oil was purified by flash chromatography on silica gel (dichlorometahne/methanol=200:5) to get 0.92 g of DO3A-*tert*-butyl.HBr. Yield is 77 %. m. p. 178-180 ˚C, R_f = 0.35 ,^{1}H NMR (in $CDCl_3$, 400 MHz): δ 10.04 (brs, 1H, N-H), 3.38 (s, 4H, CH_2), 3.30 (s, 2H, CH_2), 3.11 (s, 4H, CH_2), 2.93–2.88 (m, 12H, CH_2), 1.46 (s, 27H, CH_3); ESI-MS: *m*/*z* 514.4 M^{+}.

Synthesis of methyl salicylate or wintergreen oil

To a stirred solution of salicyclic acid (6.9 g, 50 mmol) in 75 mL of methanol (59.32 g, 1.85 mol) under ice cooling was added $SOCl_2$ (5.45 mL) dropwise over 0.5 h. After stirring the reaction mixture for 3 h, methanol was distilled out and 125 mL of distilled water was added. The separated ester was extracted with ethyl acetate (75 mL) and then washed with

50 mL of saturated sodium bicarbonate solution. Drying (with Na_2SO_4) and then removal of the solvent on rotary evaporator gave 6.2 g of Methyl salicylate or wintergreen oil in pure form. Yield is 74 %. ^{1}HNMR (in $CDCl_3$, 400 MHz): δ 10.79 (1H, s, OH), 7.82 (1H, d, J = 8 Hz, Ar-H), 7.44 (1H, t, J = 7.8 Hz, Ar-H), 6.98 (1H, d, J = 8.4 Hz, Ar-H), 6.87 (1H, t, J = 7.6 Hz, Ar-H), 3.92 (3 H, s, CH_3).



Scheme 1 – Synthesis of Gd-DO3A-MEB.

Synthesis of 2-(2-bromoethonoxy)-benzoic acid methyl ester

A mixture of 1,2-dibromoethane (39.50 g, 210 mmol), salicyclic acid methyl ester (4 g, 26.31 mmol) and Potassium Carbonate (3.63 g, 26.31 mmol) in 25 mL dry acetonitrile was refluxed for 6 h. After cooling an excess of reagents and solvents was removed under reduced pressure. To the residue methylene chloride (50 mL) was added. Inorganic salts were filtered off. The filtrate was concentrated under reduced pressure and the obtained oil was purified by column chromatography using petroleum ether and dichloromethane. Finally 2.9 g (11.20 mmol) of 2-(2-bromoethonoxy)-benzoic acid methyl ester was obtained. Yield is 43 %. ^{1}HNMR, (400 MHz, DMSO) δ 7.6 (d, Ar-H), 7.55 (t, Ar-H), 7.18 (d, Ar-H), 7.08 (t, Ar-H), 4.38 (t, 2H, OCH_2), 3.80 (s, 3H, OCH3), 3.69 (t, 2H, CH_2Br)

Synthesis of 1, 4,7-tris(*tert*-butoxycarbonylmethyl)-10-(methyl, 2-ethoxybenzoate)1,4,7,10- tetrazacyclododecane (DO3A-tert-butyl-MEB)

DO3A-*tert*-butyl.HBr (300 mg, 0.57 mmol) was dissolved in a mixture of anhydride acetonitrile (15 mL) and potassium carbonate (533 mg, 3.86 mmol). 2-(2-Bromoethonoxy)-benzoic acid methyl ester (531 mg, 2.05 mmol) in 5 mL acetonitrile was added dropwise to this mixture at 0^{o}C. Then the mixture was stirred for about 12 h at 70^{o}C under the protection of N_2. After removing the excess of K_2CO_3 by filtration, acetonitrile was evaporated in vacuum at 40^{o}C and the obtained oily product was purified by column chromatography using mixture of dichloromethane and methanol as eluent. Finally 230 mg (0.33 mmol) of DO3A-*tert*-butyl-MEB was obtained as a yellow gum. Yield is 57.5 %.^{1}HNMR (400 MHz, $CDCl_3$) δ 7.81 (t, Ar-H), 7.43 (d, Ar-H), 7.13 (d, Ar-H), 7.0 (t, Ar-H), 3.87(s, 3H, OCH_3), 4.2-2.3 (a set of very broad and multiple peaks with an integration corresponding to 26 methylene protons), 1.38 (s, 9H, CH_3), 1.28 (s, 18H, CH_3)

Synthesis of 1,4,7-*tris*(carboxymethyl)-10-(methyl,2-ethoxybenzoate)-1,4,7,10-tetraazacyclododecane (DO3A-MEB)

DO3A-*tert*-butyl-MEB (220 mg, 0.317 mmol) was dissolved in 18 mL of dichloromethane: trifluoroacetic acid at 1:2 (V:V). This solution was stirred at room temperature for about 5 h. After removing the solvent, the residue was dissolved in 0.6 mL methanol. Diethyl ether (12 mL) was added. DO3A-MEB appeared as white precipitate which was then collected and dried by a vacuum pump. (150 mg, 0.286 mmol) yield is 90 %. ^{1}HNMR (400 MHz, DMSO) δ 8.14 (d, Ar-H), 8.00 (t, Ar-H), 7.56 (d, Ar-H), 7.51 (t, Ar-H), 4.20 (s, OCH_3), 4.84-3.48 (a set of very broad and multiple peaks with an integration corresponding to 26 methylene protons).

Synthesis of the complex Gd-1,4,7-*tris*(carboxymethyl)-10-(methyl,2-ethoxybenzoate)-1,4,7,10-tetraazacyclododecane (Gd- DO3A-MEB)

$GdCl_3.6H_2O$ (280 mg, 0.96 mmol) was dissolved in 5 mL of water. Then ligand DO3A-MEB (140 mg 0.267 mmol) was added under severe stirring conditions. Sodium hydroxide solution (0.1 M) was used to adjust the solution's pH value (6-7) during the reaction. The reaction was carried out for 24 h at room temperature. Then a mixture solution of ethanol/diethyl ether (V:V=1:2) was added to the reaction mixture to precipitate the product. The solid material was collected. It was washed 3 times each with a mixture solution of ethanol/diethyl ether and ethanol. Finally the product was washed 2 times with diethyl ether. After drying a solid white compound (110 mg, 0.266 mmol) was obtained. Yield is 58 %.

CONCUSIONS

Cyclen is an aza analogue of the crown ether. It is widely used for the design and synthesis of novel ligands. In this work, cyclen was synthesized via Richman and Atkins method. It was then used to prepare DO3A-*tert-butyl.*HBr**.** This protected form of DO3A is a ligand by itself and also a key intermediate in the synthesis of MRI contrast agents. A novel ligand DO3A-MEB was synthesized from DO3A-*tert*-butyl.HBr via a series of chemical reactions. Gadolinium complex of this ligand was synthesized and its relaxation performance was determined. The complex showed 128 % enhancement compared to that widely used MRI contrast agent, Gd-DTPA. Since this complex was synthesized from cyclen derivatized ligand, therefore, it is expected to be useful for MRI technique.

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