photon emission computed tomography；SPECT）などの放射線や光子を応用した方法*1，④核磁気共鳴画像診断法（magnetic resonance imaging；MRI）などの電磁波による三次元画像診断法がある[2]．

近年，わが国における死因第 1 位は悪性新生物（がん）である[3]．心疾患や脳血管疾患などの循環器系の疾患（心臓病，脳卒中，動脈瘤，血管梗塞など）も死に至る重大な危険性があり，死因の第 2 位である．図 1 を見ると，がんによる死者の割合のみが増加の一途を辿っており，わが国の「第 3 次対がん 10 か年総合戦略」では，研究，予防および医療を総合的に推進することにより，がんの罹患率と死亡率の激減を目指すことが謳われている[4]．この第 3 次対がん 10 か年総合戦略を達成するためには，死因の上位を占めるがんや循環器系の疾患を早期に発見して早期に治療することが必要である．そのための医療技術として，画像診断法の革新は最重要課題の一つである．

医用画像診断技術は，1960 年代に入って急激な発展を見た．これはエレクトロニクス技術の進歩に負うところが大きく，コンピュータが発展し画像処理に関する新しい技術が生まれ，さらに画像診断装置の進歩などと相まって，画像診断技術が飛躍した．近年，より高度な社会ニーズ・医療ニーズが生まれ，より高感度で明瞭，鮮明かつ特異的，選択的な画像を得るために造影剤の改良が活発に行われている[5]．ここでは，画像診断法として高エネルギーの放射線被曝侵襲性をもつ X 線（X 線 CT 法）や放射性同位元素を用いる PET，SPECT ではなく，低侵襲性のラジオ波を用いたより安全な画像診断法である MRI および MRI 造影剤の開発について解説する．

MRI の原理

MRI は，核磁気共鳴を利用して生体の内部を画像化する手法である．体を磁場中に置いてラジオ波（radio frequency wave；RF 波）を当て，体内に含まれる水や脂肪のプロトンを核磁気共鳴させる．すると RF 波をかけたあとでプロトンが本来の方向にもどる際に MR 信号（磁気共鳴信号）を発する．生体組織の違いによる MR 信号の変化をコンピュータで解析処理し画像化を行う[6]（図 2）．MRI 自身の基本原理は化合物の構造解析に用いる NMR（nuclear magnetic resonance）と同じだが，計測された MR 信号をコンピュータで解析処理することで MR 画像（磁気共鳴画像）が得られる．

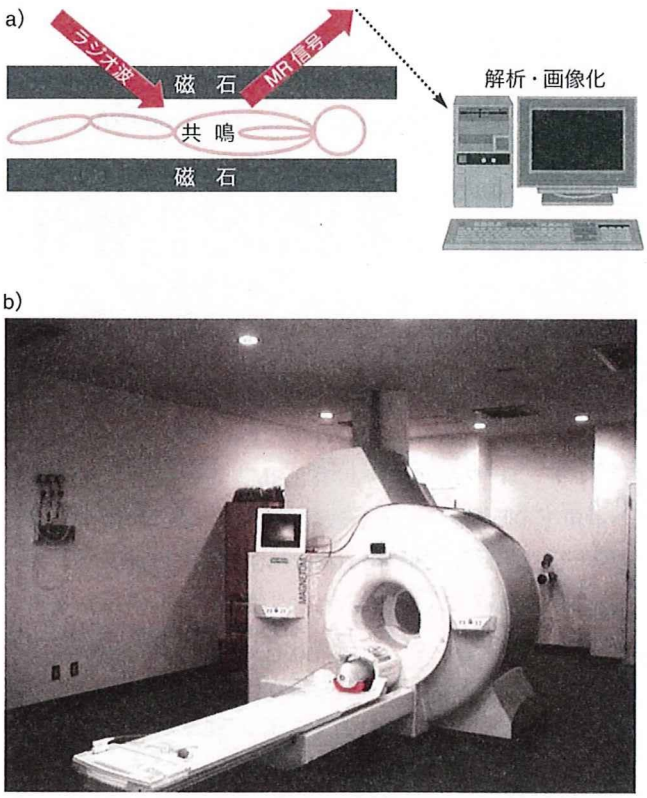


図 2　MRI の原理
a）MRI の模式図[6]，b）医療用 MRI 装置の外観．

また，高磁場の MRI 装置を用いることで，わずかな組織の違いに鮮明な画像コントラスト差を付けることが可能となり，分解能を高めることができる．さらに，目的に合わせた MRI 造影剤を使用することで，X 線 CT では画像化しにくい部位である骨に包まれた脳（骨は X 線を大幅に遮断するので，内部変化を読み取りにくい）[7]や椎間板ヘルニアの診断，生体内の代謝過程にかかわる生化学的な情報，あるいは血管や臓器などの生体内の立体的な画像情報が得られる．MRI 造影剤によって，臓器や組織の画像をより高感度で鮮明に描出することができるようになり，血管やがんの造影を可能にするなどの重要なメリットがある[8]．

一般に普及している装置と造影剤の特徴

MR の進歩はコンピュータの飛躍的発展に負うところが大きい．コンピュータを除く装置本体の開発の傾向として，現在一般的に用いられている超電導磁石の磁場強度が 1.5 ～

*1 PET は，陽電子反 β 崩壊する核種で標識された化合物を放射性トレーサーとして用いる陽電子検出を利用したコンピュータ断層撮影技術であり，SPECT は，体内に投与した放射性同位体（PET と異なり，一般の放射性同位体）から放出される γ 線を検出し，その分布を断層画像にしたものである．CT や MRI がおもに組織の形態を観察するための検査法であるのに対し，PET や SPECT は生体の機能を観察することに特化した検査法である．

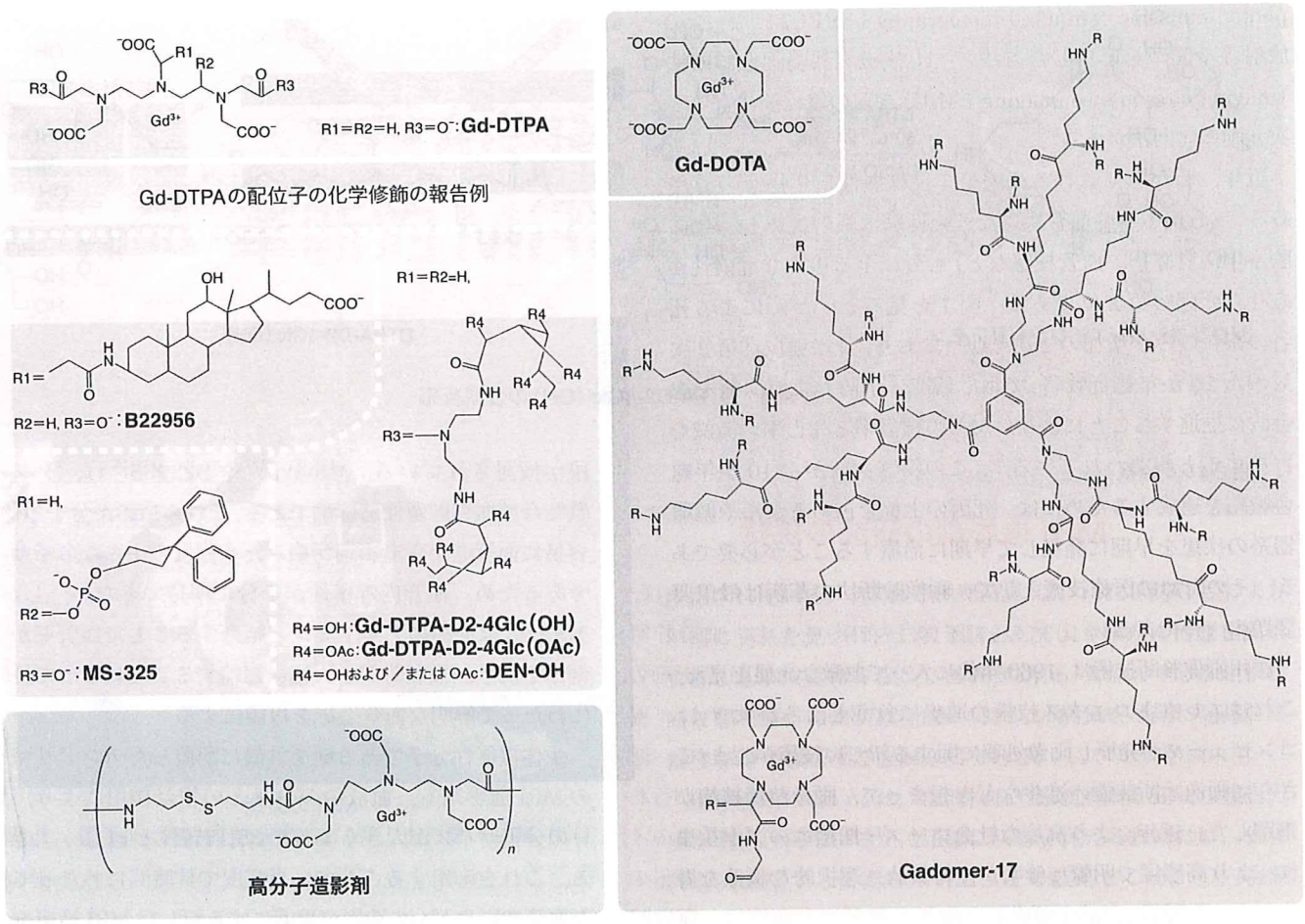


図3　おもなMRI造影剤のGd錯体の構造式

3 Tの装置から，さらに強い高磁場(7 T)化を目指した研究がさかんに行われている．現在の1.5～3 TのMRI装置では，造影剤としてガドリニウム(ランタノイド系の元素；Gd)錯体が使われ，その錯体に配位する水(H_2O)のプロトンの縦緩和(T1)を用いている．一方，7 TのMRI装置では横緩和(T2)を用いることになり，造影剤としてはナノサイズの酸化鉄微粒子を中心とした研究が行われている．

現在臨床的に使用されているMRI造影剤のGd錯体としては，最も汎用されているGd-DTPA(商品名Maginevist)のほかに，Gd-DOTA(図3)，Gd(DTPA-BMA)などがある[*2]．Gd造影剤は，Gd(Ⅲ)イオンが示す強い毒性を低減するために，配位子との非常に安定なGd錯体を形成し安全性を高めた構造が基本骨格となっている．造影効果を上げるための要素は，①Gdに配位する水分子の交換速度の上昇と安定化，②造影剤に配位する水分子の数の増加，③Gd錯体の移動および回転の抑制，などである．

Gd-DTPAの利点は，Gdによる水のプロトンの緩和効果が大きく[9]，錯体生成定数K〔Gd-DTPAでは，$\log K = 22.46$ (25 ℃)〕[10]がきわめて大きく，中性付近ではたいへん安定な錯体で副作用が少ない非常に安全な医薬品である．しかし，分子サイズが小さいために，容易に血管壁を通過して血管内貯留時間が短いという欠点(血管外漏出性)をもち，血管造影(magnetic resonance angiography；MRA)において鮮明な画像を長時間得ることが難しい，つまり“Imaging Window”が狭いのが欠点である．そのため，Gd-DTPAを造影剤として用いる場合は，所望の血管造影を行う際に海外では2～3回造影剤を投与することもある．投与量が増加することによる副作用として，とくに腎臓疾患をもつ患者の造影剤障害(腎性全身性線維症，nephrogenic systemic fibrosis；NSF)が近年報

＊2　DTPA；diethylenetriamine-N,N,N′,N″,N″-pentaacetic acid, DOTA；1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid, DTPA-BMA；DTPA-Bis(methylamide).

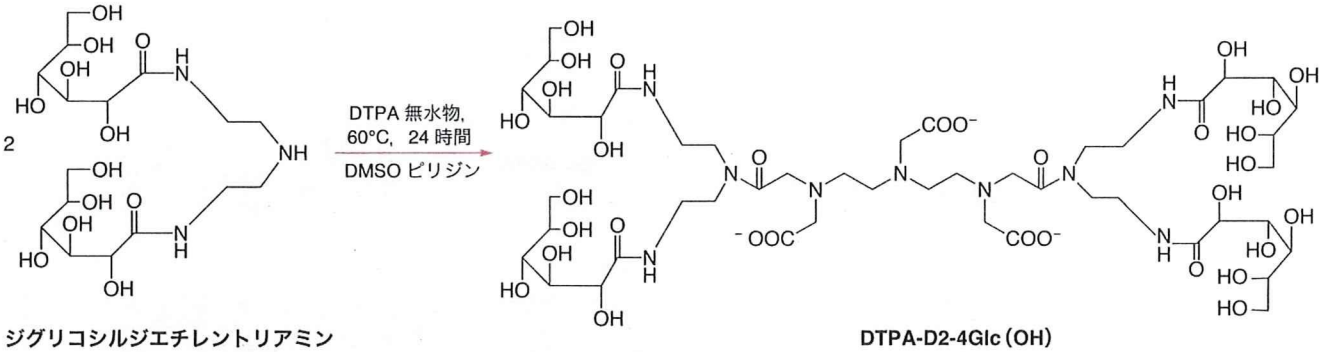


図 4　DTPA-D2-4Glc (OH) の合成反応

告されている.

MRI 造影剤の研究の現状

医療現場において，現在使用されている造影剤（たとえば，Gd-DTPA）の約 10 倍の r1 値（縦緩和率）をもち，血管貯留性が大きくてがん組織集積性の安全で新しい MRI 造影剤の開発を筆者らは目標としている．この開発に成功すれば，現在の医療現場で広く使われている普及型の装置によって，低濃度の Gd 錯体で安全な血管造影とがん検診が普遍的に行われ，血管の病気やがんの早期発見・早期治療の医療技術のイノベーションが実現すると期待される[11]．そのような背景のもと，現在の MRI 造影剤に関する研究の方向性は，血管貯留性や緩和率を高め，使用量を少なくし，高感度でより安全な造影剤をつくることである．その方法として，配位子にさまざまな組織認識部位を導入したり，あるいは配位する水分子数を増加させるなどの研究が進められている．

造影効果を高めるために，造影剤の分子量を増加させて水分子の交換を安定化させ，かつ物理的に血管壁からの漏出を防ぐ検討がなされている．分子量を大きくする手段として，造影剤のデンドリマー構造（たとえば Gadomer-17[12]，図 3）や多糖類[13]，タンパク質などの高分子を側鎖として配位した高分子血管貯留性造影剤が合成されている．分子が大きくなることで，従来の造影剤に比べて優れた血管貯留性と造影効果を示すが，分子量が非常に大きいので，MRI 造影に必要な Gd 濃度を確保するために造影剤の投薬量を多くする必要がある．この欠点を改良するため，Gd-DTPA 単位を繰り返して投薬量を多くしない長鎖分子の設計と合成例が報告されている（図 3）[14]．

造影剤の分子量を大きく増加させることなく鮮明な画像を得る方法として，血中のタンパク質と造影剤を結合させる手段が検討されている．MS-325[15] や B22956[16] は，タンパク質結合型血管貯留性造影剤である．これら造影剤分子単体は，容易に血管壁から造影剤が染みだす程度の小さな分子サイズであるため，血管内貯留性が十分に期待できない．しかし，MS-325 は血中のアルブミンと結合することで血管壁からの漏出を防ぎ，緩和時間を大幅に短縮することによって長時間にわたって鮮明な血管造影を可能にする．

生体機能性分子である糖を外殻に配置したデンドリマー型の MRI 造影剤も，血液中の分子との相互作用により，見かけの分子サイズが大きくなって血管貯留性と r1 値が大きくなる．これを応用することで，高感度で長時間にわたって鮮明な血管造影やがんの造影を可能にする新しい MRI 造影剤も報告されている[11, 17]．シュガーボールデンドリマー型の MRI 造影剤は，外殻部の糖の種類と数，コア部と外殻部間のリンカーのバリエーション，合成プロセスの開発などが行われ，さまざまな化学的修飾が可能である（図 3）．筆者らは，Gd-DTPA の欠点を解決するために，糖を外殻に配置したシュガーボールデンドリマー型の新しい Gd-DTPA 錯体を開発した[11]．たとえば図 4 のように，グルコースを 4 個外殻に配置した Gd-DTPA-D2-4Glc (OH) や，そのパーアセチル化グルコースの加水分解成績体 DEN-OH（図 3）といった Gd 錯体がある．がんは，細胞が増殖する際に多量の血液を必要とし，栄養を得るために自ら新生血管をつくるので血管が集中している．血管貯留性 Gd 錯体は，鮮明な血管造影を可能にするだけでなく，血液の豊富な臓器を鮮明に描出し，動脈側の新生血管が集中しているがん組織や血液の漏出している箇所の描出を可能にすると考えられる．そのためこれらの新しい Gd 錯体を調製して，血管造影や早期の小さな肝細胞がんを画像化し，より高感度で鮮明に描出できる造影剤の開発を目指して研究を行っている[17]．

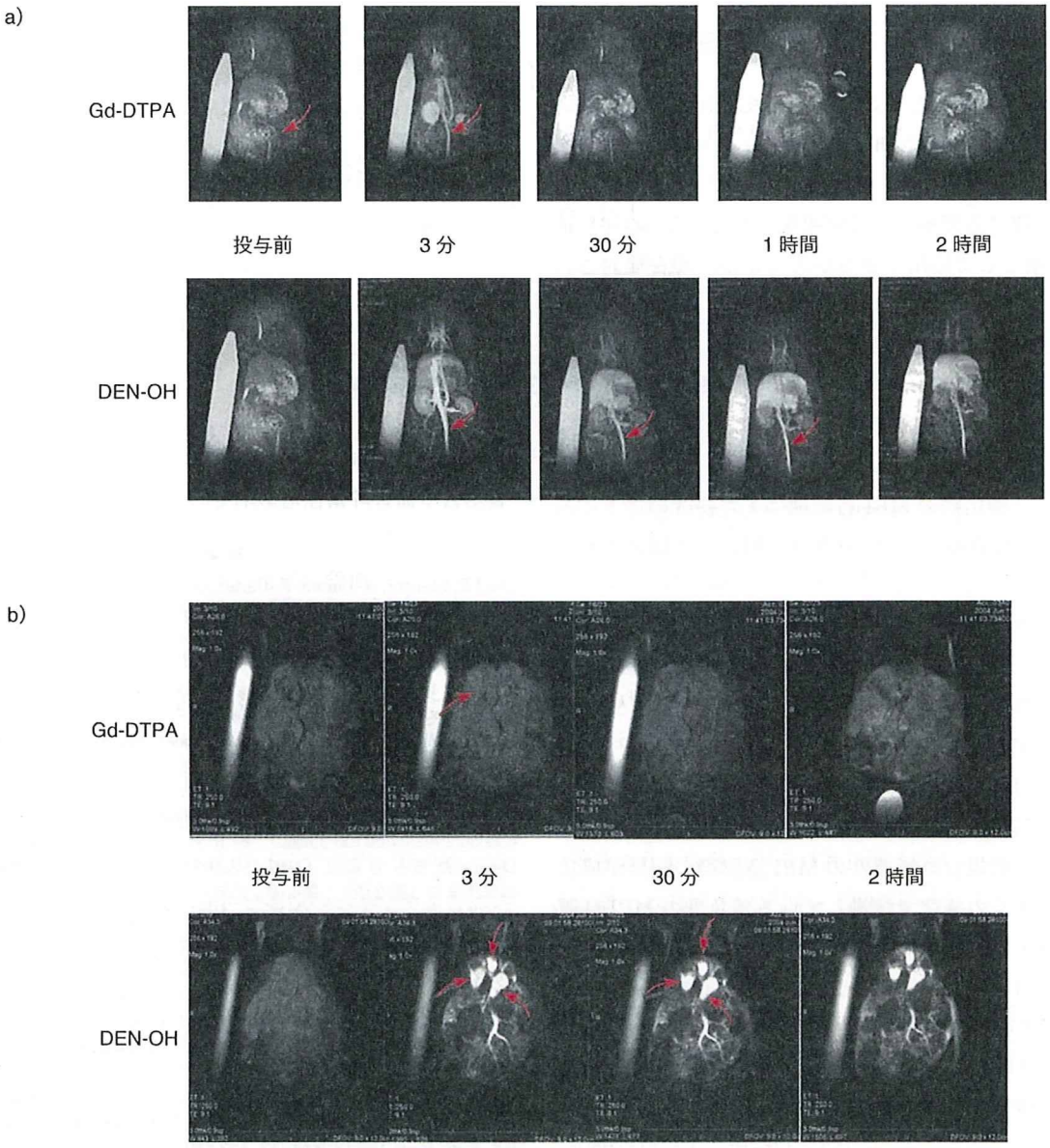


図5　Gd-DTPA と DEN-OH のラットによる *in vivo* 評価
a）血管造影，b）肝細胞がん造影．

筆者らが開発中の糖デンドリマー型の造影剤 DEN-OH は，*in vitro* 評価において高い r1 値を示している．また，*in vivo* 評価においても長時間にわたる血管貯留性を示し，鮮明な血管造影および肝細胞がんの画像化に成功した．ラットを使った DEN-OH と Gd-DTPA との比較画像を見ると，図 5 (a) において血管造影に対する DEN-OH の明らかな優位性を示している．Gd-DTPA では，造影剤の経静脈投与後 3 分程度の描出はできるが，そのあとの血管造影は難しい．一方，DEN-OH では，投与してから 2 時間経過しても血管造影が可能である．また，肝細胞がんの描出 (図 5b) については，Gd-DTPA ではほとんど描出できていないがん組織を，DEN-OH は鮮明に高感度で描出できている．このような Gd-DTPA 錯体が実用化され，がんの早期発見が普及型の MRI 装置により普遍的に行われることになれば，がんによる死亡率を激減することが可能になり，前で述べた「第 3 次対がん 10 か年総合戦略」が達成できると考えられる．

人体への安全性を重視した研究開発

金属イオンを用いない新たな造影剤として，微細な泡（microbubbles）を用いた造影剤がある．このマイクロバブル造影剤については，超音波診断機（超音波エコー）を用いて血管造影診断を行う際に造影剤として実用化されている Sono Vue を MRI 造影剤として応用できるかどうかが，現在検討されている[18]．

また，先述の高磁場の MRI 装置で用いられる横緩和（T2）強調型の酸化鉄造影剤をシクロデキストリンなどの糖鎖で包接したデキストラン酸化鉄造影剤では，E-セレクチン（糖などとの接着因子）に認識されることで血管外への漏出性を改善している[19]．酸化鉄の MRI 造影剤はまだ研究の途上にあり，MRI 装置の高磁場（7T）化の方向と相まって開発されつつある．

しかし，装置の高磁場化は人間が 7T という非常に磁場強度の高い環境に置かれることになり，未知の危険をはらんでいる．それよりも，現在一般に使用されている安全な MRI 造影剤の Gd-DTPA を基本骨格として，その欠点を補って緩和率 r1 が Gd-DTPA（r1= 約 3.5 $mM^{-1} \cdot s^{-1}$）の約 10 倍の Gd-DTPA 錯体誘導体を開発することのほうが現実性を感じる．このような新規な高機能性の MRI 造影剤の開発が成功すれば，現在多くの病院で稼働している普及型の MRI 装置でも，低濃度・少量の造影剤で血管造影やがん細胞の造影，あるいは選択的・特異的な臓器や病巣の画像診断が可能になるだろう．日本のどこにおいても，安全で安心な高いレベルの画像診断を受けることが可能となり，医療の均てん化を実現できると期待している．

●

優れた機能を備えた MRI 造影剤は，「安全・安心」な MRI の価値を一層高め，MRI が普遍的な画像診断法としてがんや各種疾患の早期発見を可能にする医療技術の革新をもたらすと期待されている．日本人は，約 3 人に 1 人ががんで亡くなっており，がんは非常に危険な病気であることは明らかである．また，約 2 人に 1 人ががんに罹病するといわれているので，誰もが罹病しうる疾患である．それゆえ，社会と医療のニーズに合った新規な高機能性の MRI 造影剤を開発し，従来の医療技術のイノベーションを図り，MRI の性能を大幅に向上させることが急務である．筆者らは，ここに述べたような MRI 造影剤を実用化することにより，がんや循環器疾患などの病気の早期発見・早期治療が今以上に容易になり，多くの人の命が救われ，また患者の QOL（quality of life）の向上が達成されることを願っている．

謝辞：新規な MRI 造影剤の開発に関して，浜松医科大学放射線教室の阪原晴海教授および竹原康雄准教授に深謝する．

参考文献

1) a) R. Manser, A. Dalton, R. Carter, G. Byrnes, M. Elwood, D. A. Campbell, *Lung Cancer*, **48**, 171 (2005). b) R. Parkesh, W. Gowin, T. C. Lee, T. Gunnlaugsson, *Org. Biomol. Chem.*, **4**, 3611 (2006). 2) a) Y. Qiao, Y. He, S. Zhang, G. Li, H. Liu, J. Xu, X. Wang, C. Qi, C. Peng, *Bioorg. Med. Chem. Lett.*, **19**, 4873 (2009). b) D. M. Goldenberg, E. A. Rossi, R. M. Sharkey, W. J. McBride, C.-H. Chang, *J. Nucl. Med.*, **49**, 158 (2008). 3) P. N. Leigh, A. Simmons, S. Williams, V. Williams, D. Brooks, *Amyotrophic Lateral Sclerosis*, **3**, 75 (2002). 4) 厚生労働省，「主な死因別にみた死亡率の年次推移」(http://www.mhlw.go.jp/toukei/saikin/hw/jinkou/geppo/nengai03/deth.html). 5) 厚生労働省，「第 3 次対がん 10 か年総合戦略」(http://www.mhlw.go.jp/houdou/2003/07/h0725-3.html). 6) R. H. Hashemi, W. G. Bradley, Jr., C. J. Lisanti 著，荒木 力 監訳，『MRI の基本パワーテキスト――基礎理論から最新撮像法まで（第 2 版）』，第 2 章，メディカル・サイエンス・インターナショナル (2004). 7) 永井輝夫，『MRI 診断学　基礎と臨床』，第 1, 2 章，朝倉書店 (1993). 8) T. Barrett, H. Kobayashi, M. Brechbiel, P. L. Choyke, *Eur. J. Radiol.*, **60**, 353 (2006). 9) R. B. Lauffer, A. C. Vincent, S. Padmanabhan, T. J. Meade, *J. Am. Chem. Soc.*, **109**, 2216 (1989). 10) A. E. Martell, R. M. Smith, "Critical Stability Constants," Vol.1, Plenum Press, New York (1974), p.282. 11) 厚生労働科学研究費医療機器開発推進研究事業，「がんを安全・高感度で鮮明に画像化できるナノサイズシュガーボールデンドリマー型新規 MRI 造影剤の開発研究」（研究代表者：静岡大学　山下光司）. 12) a) B. Misselwita, H. Schmitt-Willich, W. Ebert, T. Frenzel, H.-J. Weinmann, *Mag. Res. Mat, Phys., Biol., Med.*, **12**, 128 (2001). b) B. Misselwitz, H. Schmitt-Willich, M. Michaelis, J. J. Oellinger, *Invest. Radiol.*, **37**, 146 (2002). 13) W. A. Gibby, A. Bogdan, T. W. Ovitt, *ibid.*, **24**, 302 (1989). 14) Y. Zong, J. Guo, T. Ke, A. M. Mons, D. L. Parker, Z.-R. Lu, *J. Control. Release*, **112**, 350 (2006). 15) R. B. Lauffer, D. J. Parmelee, S. U. Dunham, H. S. Oullet, R. P. Dolan, S. Witte, T. J. McMurry, R. C. Walovitch, *Radiology*, **207**, 529 (1998). 16) C. de Haën, P. L. Anelli, V. Lorusso, A. Morisetti, F. Maggioni, J. Zheng, F. Uggeri, F. M. Cavagna, *Invest. Radiol.*, **41**, 279 (2006). 17) a) M. Takahashi, Y. Hara, K. Aoshima, H. Kurihara, T. Oshikawa, M. Yamashita, *Tetrahedron Lett.*, **41**, 8485 (2000). b) G. Yu, M. Yamashita, K. Aoshima, M. Kobayashi, T. Kato, N. Kamikage, M. Takahashi, H. Takayanagi, T. Oshikawa, S. Laurent, C. Burtea, E. L. Vander, R. N. Muller, Y. Takehara, H. Sakahara, *Heterocyclic Commun.*, **13**, 101 (2007). c) G. Yu, M. Yamashita, K. Aoshima, M. Takahashi, T. Oshikawa, H. Takayanagi, S. Laurent, C. Burtea, E. L. Vander, R. N. Muller, *Bioorganic & Medicinal Chemistry Lett.*, **17**, 2246 (2007). d) N. Miura, M. Yamashita, "Preparation of monosaccharide- and oligosaccharide-containing gadolinium compounds and contrast medium for MRI," PCT Int. Appl. (2008), p.121. 18) J. S. Cheung, A. M. Chow, H. Guo, E. X. Wu, *Neuroimage*, **46**, 658 (2009). 19) H. W. King, L. Josephson, A. Petrovsky, R. Weissleder, A. Bogdanov, Jr., *Bioconjug. Chem.*, **13**, 122 (2002).

SYNTHESIS AND EVALUATION OF NOVEL MRI CONTRAST AGENTS OF CHEMICALLY MODIFIED GD-DTPA COMPLEXES WITH SUGARS

Masaki Sugiyama, Mitsuji Yamashita, Gang Yu, Michio Fujie, Keisuke Ogawa, Nobuhisa Ozaki, Takashi Aoki, Sayaka Mizuno, Shingo Okada, Kentarou Tachi, Kengo Aoshima, A. Uma Ravi Sankar, Bitragunta Siva Kumar, Yasuo Takehara, Harumi Sakahara

Abstract:

MRI is one of medical diagnostic imaging technologies that can draw the cross section in the body. To obtain a clearer image, Gd complexes are often used as MRI contrast agents. Gd-DTPA (Gd-Diethylenetriaminepentaacetate, Magnevist®) is used in particular as the MRI contrast agents. We prepared and evaluated novel MRI contrast agents that were chemically modified Gd-DTPA with sugars (represented as Gd-DTPA-Sugar) via hydrolysis route for providing specificity to target organs and tissues. Gd-DTPA-Sugar complex showed an excellent potential for the MRI contrast agent (r_1=31.2 $s^{-1}mM^{-1}$). Gd-DTPA-Sugar complexes alternatively prepared by shorter synthetic route without protection/ deprotection (hydrolysis) method showed inferior results (r_1=6.3 and 8.1 $s^{-1}mM^{-1}$) to the hydlized product.

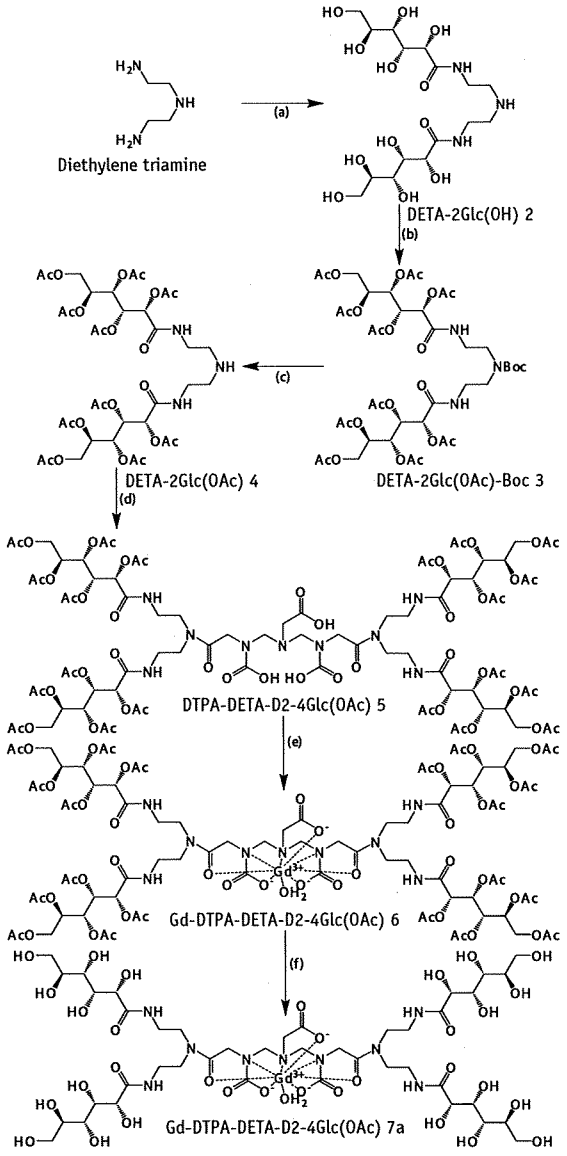
Keywords: *MRI contrast agent, Gd(III)-DTPA, tumor imaging.*

1. Introduction

MRI is one of a medical diagnostic imaging technology, and it can obtain the cross section of all angles in the body. MR imaging is obtained from the difference of nuclear relaxation time of protons, which are resonated water and fat protons in the body by irradiation in high magnetic field. Therefore, even if the contrast agents need not to be used for MRI, the imaging is possible. But to obtain a clearer image, Gd complexes are used as MRI contrast agents. Gd complexes enhance contrast by shortening T_1 relaxation times of water protons. Because T_1 relaxation time depends on Gadolinium concentration of MRI contrast agent, r_1 relaxivity that divided T_1 relaxation time by Gadolinium concentration is used as a guide to contrast intensification of MRI contrast agent. Now, Gd-DTPA is used extensively as MRI contrast agents [1]. However, Gd-DTPA has problems such as that it's not so high r_1 relaxivity, low retention in blood vessels and no specificity in the body. Our laboratory designed novel Gd-DTPA complexes for that chemistry modified sugar [2]. To give organ and tissue specificity, we focused an attention on function of sugars as organ and tissue specificity, and then chemically modified Gd-DTPA complexes with sugars became the candidates for resolving the problems of the Gd-DTPA. We prepared some Gd-DTPA-Sugar complexes and evaluated *in vivo* and *in vitro*. Gd-DTPA-Sugar complex was showed great result. So, Gd-DTPA-Sugar complex was prepared by short route for large quantity synthesis. Then, because r_1 relaxivity is improved when molecular size big, Gd-DTPA-Sugar complexes having extended carbon chains were prepared with shorter synthtic route.

2. Results and discussion

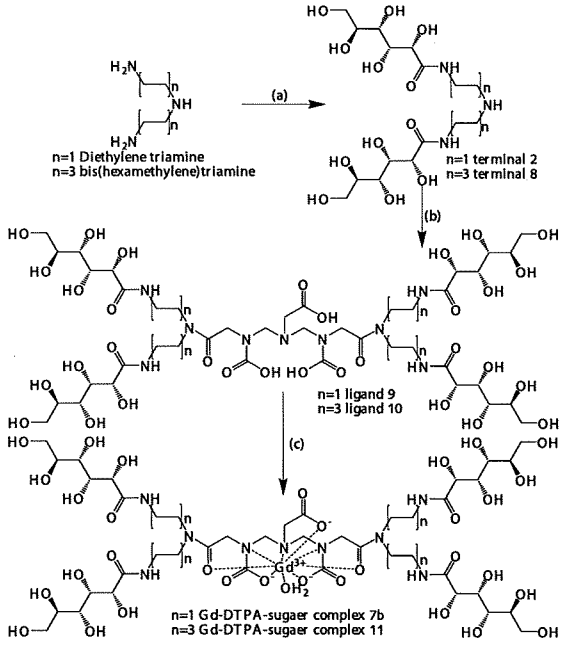
2.1. Synthesis of Gd-DTPA-Sugar complex



Scheme 1. Synthesis of Gd-DTPA-Sugar complex. Reagents and conditions: (a) D-(+)-Glucono-1,5-lactone, DMF, r.t., 24 h; (b) $(Boc)_2O$, DMF, r.t., 24 h, Ac_2O, Et_3N, r.t., 48 h; (c) TFA, CH_2Cl_2, r.t., 4 h; (d) DTPA dianhydride, DMF, Pyridine, r.t., 4 h; (e) $GdCl_3 6H_2O$, 95 °C, 1 h; (f) NaOH aq(1N), H_2O, r.t., 24 h.

A pathway of synthesis of Gd-DTPA-Sugar complex is shown in Scheme 1. Synthesis of Gd-DTPA-sugar complexes, that consist of dendrimer structure, used a convergent method. DTPA dianhydride **1** of dendrimer core was prepared by dehydration-condensation of DTPA. DETA-2Glc(OH) **2** was prepared by reaction of D-(+)-Glucono-1,5-lactone and primary amine groups of diethylenetriamine. DETA-2Glc(OAc)-Boc **3** was prepared by t-Boc protection of secondary amine group by $(Boc)_2O$ and acetylation of hydroxyl groups by Ac_2O. DETA-2Glc(OAc) **4** of dendrimer terminal was prepared by deprotection reaction of t-Boc group. DTPA-DETA-D2-4Glc(OAc) **5** of ligand was prepared by reaction of DTPA dianhydride 1 of dendrimer core and DETA-2Glc(OAc) **4.** Gd-DTPA-DETA-D2-4Glc(OAc) **6** was prepared by chelation reaction of DTPA-DETA-D2-4Glc(OAc) **5** and Gadolinium(III) ion. Gd-DTPA-DETA-D2-4Glc(OH) **7a** of Gd-DTPA-sugar complex was prepared by hydrolysis of acetyl groups of Gd-DTPA-DETA-D2-4Glc(OAc) **6** by NaOH aq(1N).

2.2. Short route syntheses of Gd-DTPA-Sugar complexes



*Scheme 2. Short route syntheses of Gd-DTPA-Sugar complexes **7b** and **11**. Reagents and conditions: (a) Ac_2O, pyridine, 65 °C, 24 h; (b) D-(+)-Glucono-1,5-lactone,DMF, **2** r.t., 24 h, **8** 80 °C, 12 h; (c) pyridine, DMSO, 60 °C, 24 h; (d) $GdCl_3 6H_2O$, pyridine, 40 °C, 12 h.*

Shorter routes of syntheses of Gd-DTPA-Sugar complexes were shown in Scheme 2. DETA-2Glc(OH) **2** and HMTA-2Glc(OH) **8** of Dendrimer terminals were prepared by reaction of D-(+)-Glucono-1,5-lactone and primary amine groups of diethylene triamine and bis(hexamethylene)triamine. DTPA-DETA-D2-4Glc(OH) **9** and DTPA-HMTA-D2-4Glc(OH) **10** of ligands were prepared by reaction of DTPA dianhydride **1** of dendrimer core with DETA-2Glc(OH) **2** and HMTA-2Glc(OH) **8** of dendrimer terminals. Gd-DTPA-DETA-D2-4Glc(OH) **7b** and Gd-DTPA-HMTA-D2-4Glc(OH) **11** of Gd-DTPA-sugar complexes were prepared by chelation reaction of DETA-D2-4Glc(OH) **9** and DTPA-HMTA-D2-4Glc(OH) **10** and Gadolinium(III) ion.

2.3. *in vitro* evaluation

The value of r_1 relaxivity, that is caluculated by being divided T_1 relaxation time by gadolinium concentration, is used as a guide to contrast intensification of MRI contrast agent, because the relaxation time depends on the gadolinium concentration of MRI contrast agent. Because gadolinium complex formation constants depend on the pH value of the aqueous media and the free gadolinium ion concentration that did not form the complexes have influence on measurements of relaxation time, the media for gadolinium complex preparation were adjusted to pH 7.0 in water, and to the media was added Chelex®100 Resin, stirred for six hours, and thus removal of the free gadolinium ion was performed. The removal of free gadolinium ion was confirmed by the color test by using Xylenol Orange. Gadolinium concentration was measured by an ICP-AES instrument because relaxation time depended on gadolinium concentration of contrast agents. T_1 was measured by TD-NMR of 0.47 T at 37 °C. T_1 was measured not only in water but also in serum albumin which is the mostly existing protein in blood.

Table 1. Comparison of r_1 relaxivity.

Gd complexes	r_1 [$s^{-1}mM^{-1}$]	
	in H_2O	in albumin
Gd-DTPA	3.5	3.5
7a	31.2	-
7b	6.3	6.8
11	8.1	7.7

2.4. *in vivo* evaluation

The relaxivity constant r_1 was calculated by the following expression.

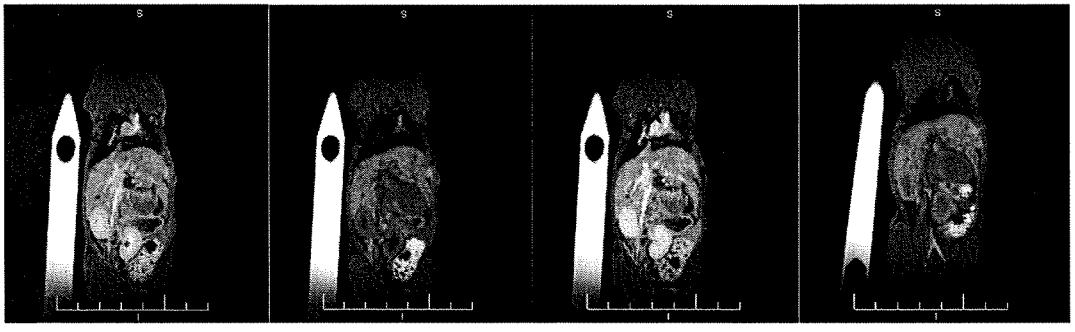


Fig. 1. Rat's MRI when Gd-DTPA (0.1 mmol/kg) was administered.

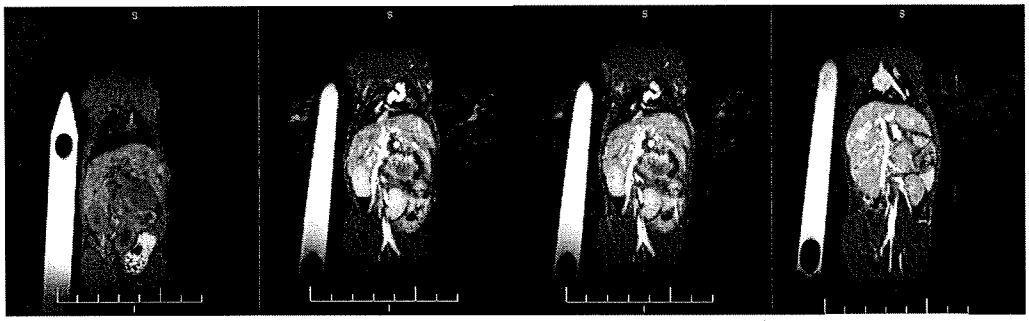


*Fig. 2. Rat's MRI when Gd-DTPA-DETA-D2-4Glc(OH) **7a** (0.05 mmol/kg) was administered.*

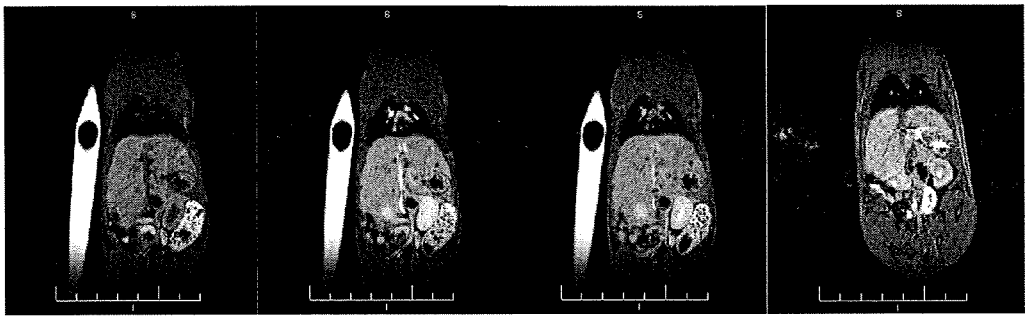


*Fig. 3. Rat's MRI when Gd-DTPA-DETA-D2-4Glc(OH) **7b** (0.05 mmol/kg) was administered.*

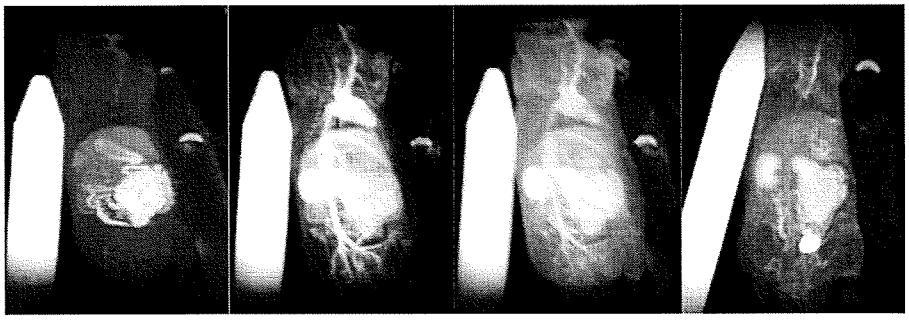


*Fig. 4. Rat's MRI when Gd-DTPA-HMTA-D2-4Glc(OH) **11** (0.05 mmol/kg) was administered.*

$$r_1 = \frac{\frac{1}{T_1} \times 1000 - r_1^{H_2O}}{[Gd^{3+}]}$$

r_1 ; relaxivity [$s^{-1}mM^{-1}$]
T_1 ; relaxation time [ms]
$r_1^{H_2O}$; water of relaxivity [$s^{-1}mM^{-1}$]
$[Gd^{3+}]$; Gadolinium concentration [mM]

The MR images of the rats were drawn by MRI machine at 3.0 T. Concentration of MRI contrast agent were adjusted by normal saline solution, Gd-DTPA solution used was 0.1 mmol/kg, solutions used for Gd-DTPA-DETA-D2-4Glc(OH) **7a** (Fig. 2), Gd-DETA-D2-4Glc(OH) **7b** (Fig. 3) and Gd-DTPA-HMTA-D2-4Glc(OH) **11** (Fig. 4) were 0.05 mmol/kg. The each MR image of Fig. 1-4 shows before administration, and then about 1, 5, and 20 minutes after the administration, respectively, from left to right.

3. Conclusion

Gd-DETA-D2-4Glc(OH) **7(a)** showed great result to give quite higher r_1 relaxivity by *in vitro*, clearer contrast effect, higher retention in blood vessels, and specificity in the liver by *in vivo*. But Gd-DETA-D2-4Glc(OH) **7(b)** being prepared by shorter route showed not so great result.

AUTHORS
Masaki Sugiyama, Takashi Aoki - Department of Materials Science, Graduate School of Engineering, Shizuoka University, Hamamatsu 432-8561, Japan.
Mitsuji Yamashita*, Nobuhisa Ozaki, Bitragunta Siva Kumar - Department of Nano-Materials, Graduate School of Science and Technology, Shizuoka University, Hamamatsu 432-8011, Japan.
E-mail: tcmyama@ipc.shizuoka.ac.jp.
Gang Yu - Innovative Joint Research Center, Shizuoka University, Hamamatsu 432-8561, Japan.
Michio Fujie - Analytical Instrument Center, Faculty of Medicine, Hamamatsu University School of Medicine, Hamamatsu 431-3192, Japan.
Keisuke Ogawa, Kengo Aoshima - Department of Materials Science, Graduate School of Science and Engineering, Shizuoka University, Hamamatsu 432-8561, Japan.
Sayaka Mizuno, Shingo Okada, Kentarou Tachi - Department of Materials Chemistry, Faculty of Engineering, Shizuoka University, Hamamatsu 432-8561, Japan.
A. Uma Ravi Sankar - Japan Association for the Advancement of Medical Equipment, Hongo, Bunkyo-ku, Tokyo 113-0033, Japan.
Yasuo Takehara, Harumi Sakahara - Radio Isotope Laboratory, Faculty of Medicine, Hamamatsu University

School of Medicine, Hamamatsu 431-3192, Japan.
* Corresponding author

References

[1] Bongrtz G., *Magn. Reson. Mater. Phy.*, vol. 20, 2007, pp. 57-62.

[2] Takahashi M., Hara Y., Yamashita M., *et.al, Tetra-hedron Lett.*, vol. 41, 2000, pp. 8485-8488.

Bioorganic & Medicinal Chemistry Letters xxx (2010) xxx–xxx

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Synthesis, in vitro and in vivo studies of Gd-DTPA-XDA-D1-Glc(OH) complex as a new potential MRI contrast agent

Nobuhisa Ozaki [a], Arigala Uma Ravi Sankar [b], Mitsuji Yamashita [a,*], Takashi Aoki [a], Yasutaka Tanaka [c], Motohiko Kimura [c], Mitsuo Toda [c], Michio Fujie [d], Yasuo Takehara [d], Harumi Sakahara [d]

[a] Graduate School of Science and Technology, Shizuoka University, Hamamatsu 432-8561, Japan
[b] Japan Association for the Advancement of Medical Equipment (JAAME), Hongo, Bunkyo-ku, Tokyo 113-0033, Japan
[c] Department of Materials Science and Chemical Engineering, Faculty of Engineering, Shizuoka University, Hamamatsu 432-8561, Japan
[d] Department of Radiology, Hamamatsu University School of Medicine, Hamamatsu 431-3192, Japan

ARTICLE INFO

Article history:
Received 21 October 2009
Revised 14 December 2009
Accepted 16 December 2009
Available online xxxx

Keywords:
Dendritic
Glycoside
Bio-distribution
Gd complexes
MRI contrast agent

ABSTRACT

A new type of dendritic molecules Gd-DTPA-XDA-D1-Glc(OH), which work as a functionalized ligand coordinating gadolinium(III) ion at the center of their frameworks with two glucose moieties on the molecular surfaces, were readily synthesized with high yield. The structures were established by IR, ^{1}H, ^{13}C NMR, and mass spectral studies. Its bio-distribution patterns were evaluated on rats.

Over the past two decades Magnetic Resonance Image (MRI) has become a very powerful tool of diagnostic medicine.[1,2] Paramagnetic materials have been investigated as MRI contrast agents (CAs). These materials enhance the contrast of the image, indirectly by remarkably shortening the magnetic relaxation time of water protons coordinated, by comparison with protons of the surrounding tissues.[3] The most frequently used CAs are stable gadolinium(III) complexes with hydrophilic poly(aminocarboxylate) ligands resulting in rapid extracellular distribution and renal elimination. Gd(III) is preferred because of its favorable magnetic properties. Gd-complexes with amphiphilic properties have previously been prepared and evaluated as blood-pool and liver imaging agents. Long chain amides and esters of Gd-DTPA (diethylenetriaminepentaacetic acid) complex are the most common.[4]

The MRI diagnosis is an excellent method to draw molecular and living body imagings, especially the strong point of the method is to be able to draw clearest 3D images among the available diagnosis. The most commonly clinically used MRI contrast agent is Gd-DTPA (Magnevist), which is one of the safest drugs. However, because of the blood vessel penetrating character of Gd-DTPA, magnetic resonance angiography by the CAs is often accompanied with narrow window, and then sometimes double or triple dose is required. To improve the week point of Gd-DTPA, we are developing chemical modification of Gd-DTPA by combining some functional residues at the outer face of the molecule.[5]

In the continuation of our work on MRI contrast agents[6] we designed a molecule (Fig. 1) to have more solubility in water. The glycoside groups have a specific target and combine with asialoglycoprotein receptor (ASGPR) on the surface of hepatocyte. Also, the glycoside groups, which were introduced into DTPA, can

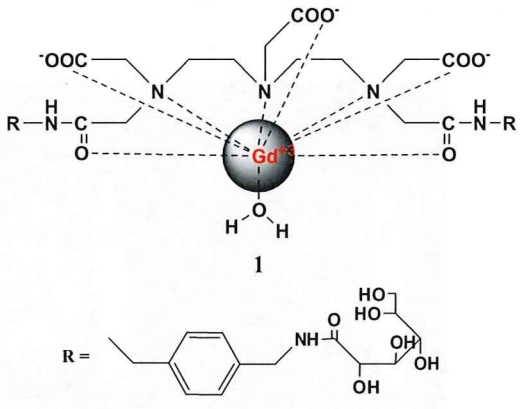
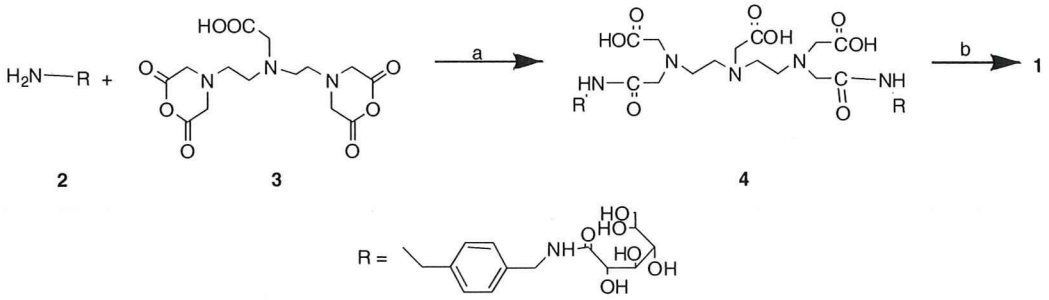


Figure 1. The structure of Gd-DTPA-XDA-D1-Glc(OH) (**1**).

* Corresponding author.
E-mail address: tcmyama@ipc.shizuoka.ac.jp (M. Yamashita).

0960-894X/$ - see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2009.12.067



Scheme 1. Reagents and conditions: (a) DMF, 60 °C, 24 h; (b) $GdCl_3$, Pyridine, H_2O, 60 °C, 24 h.

improve the water-solubility of contrast agent. So in this work, DTPA was used as a core of the ligand and glycoside was used as a biofunctional group to prepare a series of dendritic Gd-complexes for novel MRI contrast agents.

The coupling of amino glycoside branch **4**[7] and DTPA anhydride (**5**)[8] results the ligand **6**[9] which on further reaction with $GdCl_3{\cdot}6H_2O$ forms compound **1**[10] (Scheme 1).

These ligands are composed of DTPA[8] and glucose units, which may immobilize gadolinium ion at the focal points by eight coordination sites, allowing one water molecule to chelate and encapsulates the metal ions inside the glycoside clusters. Along with the 'glycoside cluster effect'[11] the carbohydrate aggregation may offer a potential advantage for site-specific delivery of the contrast agents at a molecular level since carbohydrates play significant roles in recognition processes on cell surface.[11–13] Like previous works on Gd(III)-DTPA complexes,[14,15] our new Gd-complex has showed good solubility in aqueous media, although the acetylated glycosides might reinforce their own hydrophobic features.

We developed the synthesis of new dendritic molecules and their utilization as functionalized ligands. Along with our synthetic strategy, a multi gram synthesis responsible for practical use as radiopharmaceuticals can be administered. The chelates with higher-molecular weight compounds are indispensable for prevention of their diffusion from the intravascular space during MRI examinations.[16] Accordingly; these gadolinium(III) chelates may fulfill many criteria and for superior contrast agents after creation of structural modifications. Following intensive investigations on a wide variety of carbohydrate-modified ligands, the feasibility of their metal complexes as new potentional candidates for MRI contrast media are now in progress.

In vitro evaluation. r_1 relaxivity that divided T_1 relaxation time by gadolinium concentration is used as a guide to contrast intensification of MRI contrast agent because the relaxation time depends on the gadolinium concentration of MRI contrast agent. Because gadolinium ion that did not form complexes have influence on measurements of relaxation time, they were adjusted to pH 7.0 in water, added Chelex®100 Resin, stirred for 6 h, and removed free gadolinium ion. The removal of free gadolinium ion was confirmed with color test by Xylenol Orange. Gadolinium concentration was measured with ICP-AES because relaxation time depended on gadolinium concentration of contrast agents. T_1 was measured by TD-NMR of 0.47 T at 37 °C. T_1 was measured not only in water but also in serum albumin which was most existing protein in blood. r_1 was calculated by the following expression, the results of the r_1 value and the standard deviation calculated are shown in Table 1.

Table 1
Comparison of r_1 relaxivity

Gd complexes	r_1 [s^{-1} mM^{-1}]		Standard deviation (mean ± S.D.)	
	In H_2O	In albumin	In H_2O	In albumin
Gd-DTPA	3.5	3.5	–	–
1	10.5	19.75	0.0612	0.0070

$$r_1 = \frac{\frac{1}{T_1} \times 1000 - r_1^{H_2O}}{Gd^{3+}}$$

r_1; relaxivity [s^{-1} mM^{-1}]r, T_1; relaxation time [ms], r_1; relaxivity of H_2O [s^{-1} mM^{-1}], [Gd^{3+}]; gadolinium concentration [mM].

In vivo evaluation. We imaged the MR image of the rats with MRI machine at 3.0 T. Concentration of MRI contrast agent were adjusted by normal saline solution, Gd-DTPA was 0.1 mmol/kg, Gd-DTPA-XDA-D1-2Glc(OH) (**1**) were 0.05 mmol/kg. In Figures 2 and 3, MR image in the left figure is rat before administration, and the right figures are rat after 1 min administration, 5 min after administration, 20 min after administration, respectively.

Bio-distribution of gadolinium in vivo was examined using rats with liver tumor. Gd-DTPA-XDA-D1-Glc(OH) (0.05 mmol/kg,

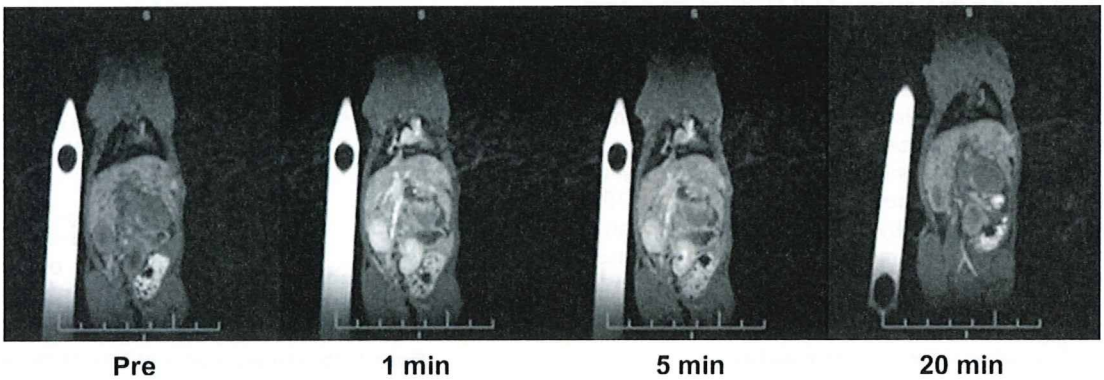


Figure 2. Rat's MRI when Gd-DTPA (0.1 mmol/kg) was administered. The time in min indicates the time after the administration of the contrast agent. The time (Pre, 1 min, 5 min, and 20 min) passes from left to right.

Please cite this article in press as: Ozaki, N.; et al. *Bioorg. Med. Chem. Lett.* (2010), doi:10.1016/j.bmcl.2009.12.067

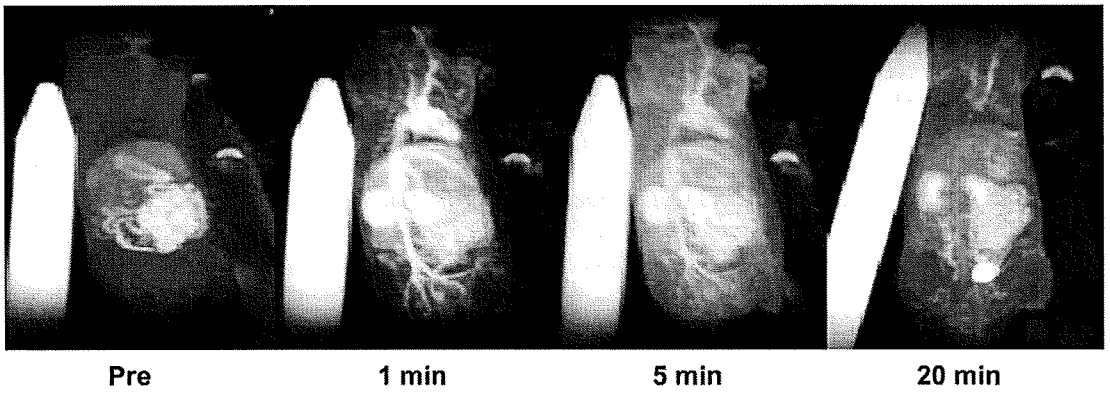


Figure 3. Rat's MRI (at Pre, 1 min, 5 min, and 20 min) when Gd-DTPA-XDA-D1-2Glc(OH) (**1**; 0.05 mmol/kg) was administered.

0.3 mL) was injected intravenously into rats (see Fig. 3). At 1 min, 5 min, and 20 min after injection, the liver, kidney, muscle, blood, pancreas, and spleen were excised. The results are shown in Figure 3, from the figure, we can find Gd-DTPA-XDA-D1-Glc(OH) displays good selectivity to liver, kidney, blood vessel, and spleen.

Figure 3 shows the bio-distribution of the Gd-DTPA complex. The gadolinium concentration of Gd-DTPA in the muscle was the same level as that of the Gd-DTPA-XDA-D1-Glc(OH). Although, the gadolinium concentration of Gd-DTPA in the kidney was higher than that of Gd-DTPA-XDA-D1-Glc(OH), the values of Gd-DTPA in the liver and blood were much lower than those of Gd-DTPA-XDA-D1-Glc(OH) and gadolinium concentration was not observed in the pancreas and spleen.

The high gadolinium concentration in liver and blood indicates the Gd-DTPA-XDA-D1-Glc(OH) has good selectivity to organs. However to compare with the values of gadolinium concentration at 2 h after injection, the values of gadolinium concentration are not changed at 24 h after injection, which indicate the Gd-DTPA-XDA-D1-Glc(OH) cannot be excreted from body timely.

The MR imaging of rats with liver tumors was shown in Figure 3. The liver tumors have been found at 2 h after injection of Gd-DTPA-XDA-D1-Glc(OH). However, the liver tumors cannot be found after injection of Gd-DTPA. The results indicate Gd-DTPA-XDA-D1-Glc(OH) possesses higher tumor-selectivity than Gd-DTPA.

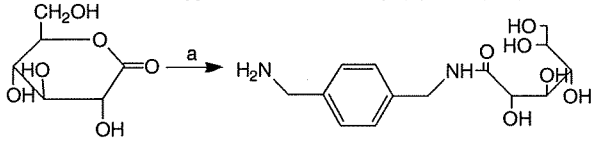
We have succeeded in the synthesis of a new gadolinium complex, Gd-DTPA-D1-Glc(OH) as a MRI CAs. The higher accumulation in the blood vessel and higher tumor-selectivity of Gd-DTPA-D1-Glc(OH) than Gd-DTPA indicates that the Gd-complex has a potential as MRI angiography and early stage findings and medical treatments for tumors.

Acknowledgements

We would like to thank the Ministry of Health, Labour and Welfare, Japan, and J.A.M.M.E, for providing financial support.

References and notes

1. Lauffer, R. B. *Chem. Rev.* **1987**, *87*, 901.
2. Tweedle, M. F. In *Lanthanide Probes in Life, Chemical, and Earth Sciences*; Bunzli, J. C. G., Choppin, G. R., Eds.; Elsevier: Amsterdam, 1989. Chapter 5.
3. Caravan, P.; McMurry, J. J.; Lauffer, R. B. *Chem. Rev.* **1999**, *99*, 2293.
4. Kabalka, G. W.; Davis, M. A.; Moss, T. H.; Buonocore, E.; Hubner, K.; Holmberg, E.; Maruyama, K.; Haung, L. *Magn. Reson. Med.* **1991**, *19*, 406.
5. Yamashita, M.; Ozaki, N.; Yamashita, J. *Kagaku (Chemistry)* **2009**, *64*, 56.
6. Takahashi, M.; Hara, Y.; Aoshima, K.; Kurihara, H.; Oshikawa, T.; Yamasitha, M. *Tetrahedron Lett.* **2000**, *41*, 8485.
7. To a solution of D-(+)-glucono-1,5-lactone (**A**,1 g, 7.3 mmol) in dry DMF(20 mL) was added *p*-xylenediamine (1.3 g, 7.3 mmol) then stirred for 24 h at rt. After completion of the reaction, the solution was purified, and the solvent was evaporated to dryness under reduced pressure to get **B** as yellow crystals with 90%. ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.10–8.05, 7.21–7.19, 4.50–4.05, 3.95–3.32, 2.88, 2.73, 2.50. ^{13}C NMR (75 MHz, $CDCl_3$); δ 44.0, 45.2, 64.6, 69.5, 71.8, 72.0, 72.6, 128.4, 135.2, 141.6 ppm. MALDI-TOF MS: *m/z* (%) = 316 (100) M^+.



A **B**

Reagents and conditions: (a) *p*-Xylenediamine, rt, 24 h.

8. Blish, S. W. A.; Chowdhury, A. H. M. S.; Mcpartlin, M.; Scowen, T. J.; Bulman, R. A. *Polyhedron* **1995**, *14*, 567.
9. The synthesis of dendritic ligand **4** employed a convergent method to couple core and glycoside branch. To the solution of terminal **2** (1.4 g, 4.4 mmol) in DMF (25 mL) was added DTPA dianhydride (**5**, 0.8 g, 2.2 mmol) and stirred for 24 h at 60 °C. After the completion of the reaction and evaporation of the solvent gave a series of ligand with two sugars **6** with 90%. ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.29–8.02, 7.95–7.02, 4.28–4.06, 3.96–3.3.15, 2.89–2.50. ^{13}C NMR (75 MHz, $CDCl_3$): δ 43.6, 44.0, 52.6, 54.8, 59.6, 59,8, 60.6, 64.6, 69.5, 71.8, 71.9, 72.4, 72.6, 128.4, 135.2, 171.4, 172.6, 173.2. MALDI-TOF MS: *m/z* (%) = 985 (100) M^+.
10. To a solution of ligand **4** (1.7 g, 1.7 mmol) in water was added pyridine (1.4 mL, 17.75 mmol) and the reaction mixture was stirred thoroughly for 10 min at rt. To this $GdCl_3{\cdot}6H_2O$ (0.80 g, 1.7 mmol) was added slowly and the reaction was kept at 60 °C and stirred for 24 h. After completion of the reaction water was removed under vacuum and the crude product was dissolved in water and the excess of Gd was removed by using Chelex® resign and checked by use of xylenol orange indicator.[17] After removal of excess Gd the resin was filtered off and after the completion of the reaction, the solvent was removed by rotary-evaporator under reduced pressure then dried to yield **1** with 90%. MALDI-TOF MS: *m/z* (%) = 1140 (100) M^+-H_2O.
11. Konings, M. S.; Dow, W. C.; Love, D. W.; Raymond, K. N.; Quay, S. C.; Rocklage, S. M. *Inorg. Chem.* **1990**, *29*, 1488.
12. Lee, Y. C.; Lee, R. T. *Acc. Chem. Res.* **1995**, *28*, 321.
13. Zanini, D.; Roy, R. *J. Am. Chem. Soc.* **1997**, *119*, 2088.
14. Kawa, M.; Frechet, J. M. *J. Chem. Mater.* **1998**, *10*, 286.
15. Newkome, G. R.; Guther, R.; Moorefield, C. N.; Cardullo, F.; Echegoyen, L.; Perez-Cordero, E.; Luftmann, H. *Angew. Chem., Int. Ed. Engl.* **1995**, *34*, 2023.
16. Formation of 1:1 Gd(III) complex was deduced on the basis of titration experiment, where 0.5, 1.0, or 10.0 equiv of Gd(III) was added relative to the ligands upon complexation.
17. Barge, A.; Cravotto, G.; Gianolio, E.; Fedeli, F. *Contrast Med. Mol. Imaging* **2006**, *1*, 184.

R&D OF NOVEL MEDICINAL MATERIALS FOR CURING CANCER: SUGAR MODIFIED GD-DTPA MRI CONTRAST AGENTS AND PHOSPHA SUGAR ANTI-CANCER AGENTS

Junko Yamashita, Mitsuji Yamashita, Michio Fujie, Kazuhide Asai Takuya Suyama, Satoru Ito, Valluru Krishna Reddy, Manabu Yamada, Keisuke Ogawa, Nobuhisa Ozaki, Satoki Nakamura, Takashi Aoki, Gang Yu, Kengo Aoshima, Tatsunori Kato, Nao Kamikage, Keita Kiyofuji, Yasuo Takehara, Harumi Sakahara, Hisao Takayanagi, Tatsuo Oshikawa, Sophie Laurent, Carmen Burtea, L. Vander Elst, Robert N. Muller

Abstract:

Novel Sugar Dendritic Gd-DTPA Complexes for MRI Contrast Agents were prepared and evaluated by in vitro and in vivo methods. The sugar dendritic MRI contrast agents have a good blood vesse pool character, and draw blood vessels and liver cancer remarkably clearer than the clinically using Gd-DTPA. Phospha sugar derivatives or phosphorus heterocyclic derivatives provided by functional groups such as epoxide, bromide, etc., were prepared and evaluated by MTT in vitro method. These phospha sugar derivatives showed excellent activities against leukemia cells as well as solid cancer cells in fashions of (i) higher activity, (ii) wider spectra, (iii) higher selectivity and specificity distingushing healthy and cancer cells, etc., compared with the molecular targeting chemotheraputic anti-cancer agent, Gleevec.

Keywords: *MRI contrast agent, sugar-ball-dendrimer, Gd (III)-DTPA complex, phospha sugars, phospholanes, anticancer agent, tumor.*

1. Introduction

Cancer is one of the most serious diseases, and the disease is expected to be more and more serious if the innovation in cancer therapy will not be realized hereafter. To innovate in cancer therapy it is very important that medicinal materials or technologies to find tumors safely at the very early stage (early diagnosis) and to cure tumors by improving the quality of life (QOL) of the patients. To develop and realize such medicinal materials, highly functionalized MRI contrast agents [1], [2], which provide clearer images of very small cancers, are required. The currently quite often used MRI contrast agent is Gd-DTPA (Magnevist) which is safe and potential MRI contrast agent, however, the MRI contrast agent has poor characters for imaging blood vessels and cancers. To improve the poor characters of Gd-DTPA to draw cancers as well as blood vessels (Magnetic Resonance Angiography; MRA), Gd-DTPA was chemically modified by sugars. The results are described in the first part of this paper.

Molecular targeting chemotherapeutic agents play one of the most important role in curing cancers. Among chemotherapeutic agents Gleevec (Imatinib) is one of the most commonly used medicines. Gleevec has potential activity against cancers, especially leukemia cells, nevertheless, Gleevec has lower activity towards some of leukemia cells. Gleevec is also used for solid tumors, however, to cure larger tumor tissues by Gleevec some times faces lower efficiency for the complete cure. Therefore, new researches to develop alternative anticancer agents to Gleevec are steadily demanded.

Phospha sugar is one of sugar analogs which have a phosphorus atom in place of the ring oxygen atom of normal sugars and is assigned to a category of *pseudo* sugars. *Phospha* sugars are not found in nature yet, and then all of them reported until now are chemically synthesized. On the other hand, the alternative pseudo sugars, such as *aza-*, *carba-*, and *thia-sugars* [3], [4], having a nitrogen, carbon, and sulfur atom, respectively, in the hemiacetal ring of sugars, are widely known in nature and are also chemically synthesized and modified extensively. Many of them are known to have important biological activities.

Sugar starting materials, which have an oxygen atom in the hemiacetal ring, are basically and usually used to prepare *pseudo* sugars. To prepare phospha sugars from sugar staring materials is rather difficult compared with the other pseudo sugars, therefore, less kinds of *phospha* sugars are prepared and a little is known about the character of phospha sugars compared with *aza-*, *thia-* sugars, etc.

As an alternative preparative method, we have developed *phospha* sugar chemistry starting from phosphorus heterocyclic compounds, e.g., 2-phospholene derivatives, by chemical modification at their reactive sites [3], [4]. Addition of bromine to the unsaturated C=C double bond of the starting 2-phospholene derivatives produced 2-bromo- or 2,3-dibromophospholane derivatives. Substitution reaction of 2-bromophospholane derivatives, which correspond to 2-bromo-2-deoxyphospha sugar derivatives, with amine nucleophiles gave *N*-glycosides of *phospha* sugar derivatives. Further, nucleic acid bases such as uracil were introduced into 2-phospholene 1-oxide derivatives by the cyclization reaction of acrylamide derivatives to prepare phospha sugar nucleosides [3], [4].

We are continuously searching biological activity for these *phospha* sugars or phosphorus heterocycles by *in vitro* and *in vivo* bio-assays. In the second part of this paper, we will deal with the successful preparation of many kinds of *phospha* sugars or phospholane derivatives from 1-phenyl-3-methyl-2-phospholene 1-oxide and the related derivative. The biological activities of the prepared *phospha* sugars or phospholane derivatives were evaluated by MTT *in vitro* method for leukemia cell. These deta will be reported in this paper.

2. Results and discussion

2.1. Sugar-Ball-Dendritic MRI contrast agents

2.1.1 Preparation of Sugar-Ball-Denderimers of Gd-DTPA-Dn-Sugar structure

Currently, one of the most often used clinical MRI contrast agent is Gd-DTPA complex (Gadolinium Diethylenetriamine pentaacetic acid: Magnevist) (Fig. 1), whose molecular size is small and then the contrast agent penetrates the blood vessel. To make the MRI contrast agent remain in the blood vessels so as to draw clearer blood vessels (Magnetic Resonance Angiography (MRA)) and tumors, sugar ball dendritic structure of Gd-DTPA complexes were designated and synthesized. These dendritic Gd-DTPA complexes are generally represented in this paper as Gd-DTPA-Dn-Sugar. The reaction to prepare Gd-DTPA-D1-Glc(OAc) (four peracetylated glucose derivative) is exemplyfied in Scheme 1 [1], [2]. The product of the alikaline hydrized Gd-DTPA-D1-Glc(OH) is represented here as DEN-OH.

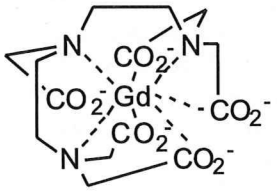
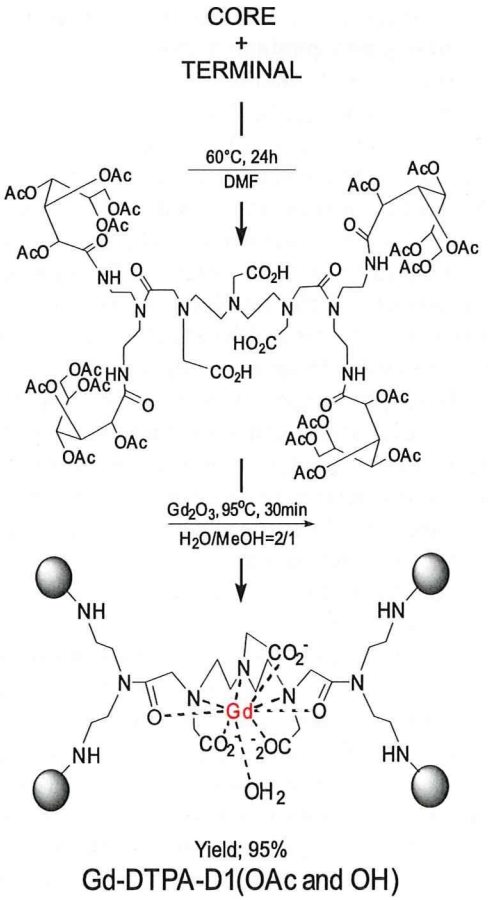


Fig. 1. Gd-DTPA (Magnevist).



Scheme 1. Preparation of Gd-DTPA-D1-Glc (OAc). The hydrized product of Gd-DTPA-D1-Glc(OAc) is simpely represented here as DEN-OH.

2.1.2 In vivo evaluation of DEN-OH as the MRI contrast agents to draw MRA and tumour

The Gd-DTPA-D1-Glc(OAc) and DEN-OH were subjected to *in vivo* evaluation by using rats. The MRA by DEN-OH drew blood vessel clearly as shown in Fig. 2 and tumors on the liver of rats were also drawn quite clear images as shown in Fig. 3.

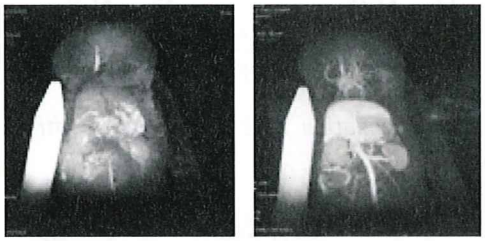


Fig. 2. MRA of rat at 30 min after injection (Left: by Gd-DTPA; Right: by DEN-OH).

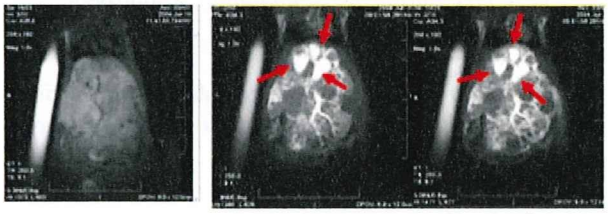


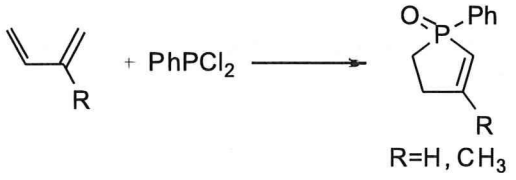
Fig. 3. MRI of liver cancer of rat (Left: by Gd-DTPA at 3 min after injection; Right two: by DEN-OH at 3 min and 30 min after injection).

Fig. 2 shows that DEN-OH draws boold vessels remarkably clearer than Gd-DTPA. And Fig. 3 shows that DEN-OH draws the liver cancer quite clearly. These results strongly indicate that Gd-DTPA-Dn-Sugar must be novel good MRI contrast agent for early stage tumor drawing.

2.2. Phospha sugar anti-cancer agents

2.2.1 Preparation of phospholenes, phosphorus heterocyclic compounds

The McCormack reaction of 1,3-dienes with phosphorus chlorides, e.g., phosphorus tichloride, phenylphosphonus dichloride, afforded 2-phospholene derivatives (Scheme 2), which were used as the starting materials of *phospha* sugar derivatives or phospholanes.

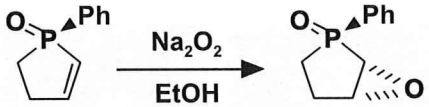


Scheme 2. Synthesis of 2-phospholenes (McCormack Reaction).

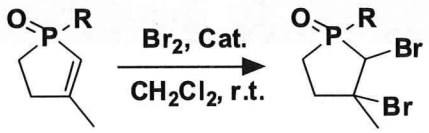
2.2.2 Preparation of *phospha* sugars or phospholanes

1,2-Anhydro-*phospha* sugars or 2,3-epoxy-1-phenylphospholane 1-oxides were prepared by an epoxidation of 2-phospholenes with sodium peroxide as shown in Scheme 3. The epoxidation reaction was stereospecific and stereoselective and gave essentially *threo* epoxide. The *threo* epoxide was defined by the two oxygen atoms

of epoxide and phosphoryl locate on the same side of the sugar ring skeleton. The 1,2-dibromo-1,2-dideoxy-*phospha* sugars or 2,3-dibromo-1-phenylphospholane derivatives were prepared by an addition reaction of bromine to the double bond of 2-phospholenes as shown in Scheme 4 [3,4].



Scheme 3. Epoxidation of 2-phospholenes with sodium peroxide.



Scheme 4. Preparation of 1,2-dibromo-1,2-didoxy-phospha segars.

The substitution, addition, homologation reactions, etc., were carried out to prepare new phospha sugars or phospholane derivatives.

2.2.3 Evaluation of phospha sugars or phospholanes by *in vitro* MTT method

MTT method of *phospha* sugars against leukemia cell lines, K562 and U937, were carried out for *in vitro* evaluation as the anti-tumor agents [5]. The results are shown in Fig. 4 and Fig. 5. Fig. 4 shows that some of phospha sugars, e.g., bromohydrin, epoxide, dibromo derivatives, were active and many of the other derivatives were inactive.

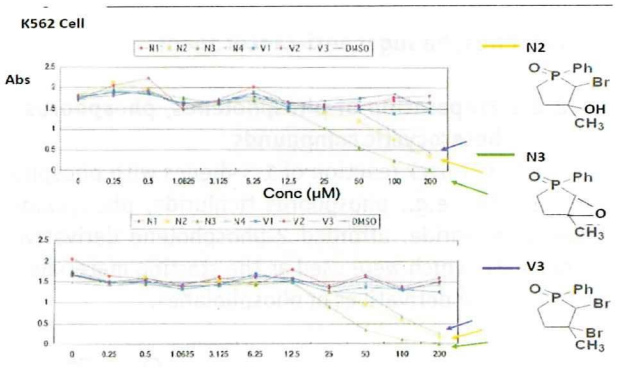


Fig. 4. MTT evaluation of phospha sugars as anti-tumor agents against K562 cells.

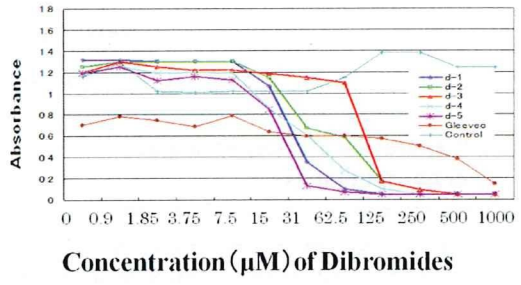


Fig. 5. MTT evaluation of phospha sugars (dibromophospha sugars) as anti-tumor agents (comparison with Gleevec) against U937 cells.

Fig. 5 shows that the dibromo derivative was also active against U937 cell lines. The diastereomers of the dibromide showed different activities, and they are much more active against the U937 cell lines than Gleevec. These findings strongly indicate that the *phospha sugars* must be quite active and wide spectral anti-tumour agents.

The dibromide showed that the *phospha* sugar were active not only against solution cancer (leukemia cells) but also against solid cancer (stomach cancer). Flow cytomertry for the preliminary mechanistic study indicated that these *phospha* sugars induced apoptosis for leukemia cells. Further studies on the optimization of the structure-activity of *phospha sugar* derivatives against cancer and the mechanistic studies are under progress.

3. Conclusion

The novel Gd-DTPA-Dn-Sugar structured MRI contrast agents could image quite small sized tumors, and then could be used for MRI contrast agents for MRA and initial stage tumor drawing. The novel *phospha* sugars could kill the leukemia cells in (i) high activity, (ii) selective and specific manner, (iii) wide spectra, by induction of apoptosis of cancer cells. Together with these novel medicinal materials, early stage findings and early stage chemotherapeutic treatment to cure cancers should be realized in the near future.

4. Experiment

4.1. Synthesis of 2,3-dibromo-3-methyl-1-phenylphospholane 1-oxide

To CH_2Cl_2 (10ml) solution of 3-methyl-1-phenyl-2-phosholene 1-oxide (0.27 g, 1.4 mmol) and Mn(IV) dioxide (0.24 g, 2.8 mmol; 2.0 eq.) was added drop wise CH_2Cl_2 (10 ml) solution of bromine (0.40 ml, 7.8 mmol; 5.6 eq.) and the reaction mixture was stirred for 8 h at room temperature. The reaction was quenched by addition of saturated sodium sulfite aqueous solution. The aqueous mixture was extracted with chloroform (10 ml x 3). The organic layer was neutralized with saturated $NaHCO_3$ aqueous solution, washed with saturated NaCl solution and dried over with anhydrous sodium sulfate. The solvent of the filtrate was evaporated under a reduced pressure to give an oily mixture of product. The mixture was purified by column chromatography on silica gel by using chloroform and methanol (30 : 1) as the eluent to give 2,3-dibromo-3-methyl-1-phenylphosholane 1-oxide (0.37 g) in 75% yield; m.p. (Shimadu Simultaneous DTA-TG Apparatus (DTG-60A50AH)) 189.20 °C; b.p. 280.24 °C; TLC (Silica gel: Wako Chromato Sheet and/or Merk Kieselgel 60; Eluent: $CHCl_3$: MeOH = 20 : 1), Rf = 0.42; MS (MALDI-TOF-MS: GL Science (Voyager-DE Porimerix); Matrix: α-Cyano-4-hydroxy-cinnamic acid (m/z)), 349.29 (M - H^+ (Molecular peak - 1); isotope peaks: 349.29, 351.29, and 353.29) and 351.29 (M + H^+ (Molecular peak + 1); isotope peaks: 351.29, 353.29, and 355.29); IR (JASCO FT/IR 410 (KBr)): 1126 cm^{-1} (P=O), 748 cm^{-1}, 1396 cm^{-1} (C-Br); ^{1}H-NMR (JEOL JNM-AL300 (300 MHz) and Hitach R90H (90 MHz); Solvent: $CDCl_3$, δ(ppm)); 1.67 (s, 3H, CH_3), 2.36-2.46 (m, 2H, H-4), 2.97-3.02 (m, 2H, H-5) 4.28-4.31 (m, 1H, C-2), 7.51-7.70 (m, 5H, Ph-H). HPLC (Apparatus:

JASCO HPLC Set (JASCO 860-CO, 880-PU, 875-UV, RI-930, and 807-IT; Column: Silica gel (Analysis: Wakopac, Wakosil Φ 4.6 mm × 250 mm, Eluent: $CHCl_3$: MeOH = 30 : 1, Flow rate: 0.5 ml/min), RT (retention time: min) values of diastereo isomers were 8.1, 9.1, 9.9, and 11.5.

AUTHORS
Junko Yamashita, Mitsuji Yamashita*, Michio Fujie, Kazuhide Asai, Takuya Suyama, Satoru Ito, Valluru Krishna Reddy, Manabu Yamada, Keisuke Ogawa, Nobuhisa Ozaki, Takashi Aoki, Gang Yu, Kengo Aoshima, Tatsunori Kato, Nao Kamikage, Keita Kiyofuji - Graduate School of Science and Technology, Shizuoka University, Hamamatsu 432-8561, Japan.
E-mail: tcmyama@ipc.shizuoka.ac.jp.
Yasuo Takehara, Michio Fujie, Harumi Sakahara, Satoki Nakamura - Faculty of Medicine, Hamamatsu University School of Medicine, Hamamatsu 431-3192, Japan.
Hisao Takayanagi - Pharmaco-chemical Reserach Center, Tanabe Mitsubishi Pharmacy, Yokohama 227-0033, Japan.
Tatsuo Oshikawa - Department of Materials Chemistry, Numazu College of Technology, Numazu 410-8501, Japan.
Sophie Laurent, Carmen Burtea, L. Vander Elst, Robert N. Muller - NMR Laboratory, Department of Organic Chemistry, University of Mons-Hainaut, B-7000 Mons, Belgium.
* Corresponding author

References

[1] Takahashi M., Hara Y., Yamashita M., *et al.*, *Tetrahedron Lett.*, vol. 41, 2000, pp. 8485-8488.
[2] H. Lammers, M. Frederic, R. N. Muller, *et al.*, *Inorg. Chem.*, vol. 36, 1997, pp. 2527-2538.
[3] Yamashita M., Reddy V.K., Rao L.N., Haritha B., Maeda M., Suzuki K., Totsuka H., Takahashi M., Oshikawa T., *Tetrahedron Lett.*, vol. 44, 2003, p. 2339-2341.
[4] Totsuka H., Maeda M., Reddy V.K., Takahashi M., Yamashita M., *Heterocyclic Commun.*, vol. 10, 2004, p. 295-300.
[5] Nakamura S., Yamashita M., Yokota D., Hirano I., Ono T., Fujie M., Shibata K., Niimi T., Suyama T., Maddali K., Asai H., Yamashita J., Iguchi Y., Ohnishi K. *Investigational New Drugs*, in press (2009).

PREPARATION AND CHARACTERIZATION OF PHOSPHOLANES AND PHOSPHA SUGARS AS NOVEL ANTI-CANCER AGENTS

Satoru Ito[a], Mitsuji Yamashita[*a], Taishi Niimi[a], Michio Fujie[a,b], Valluru Krishna Reddy[a], Hirono Totsuka[a], Buchammagari Haritha[a], Kasthuraiah Maddali[a], Satoki Nakamura[b], Kazuhide Asai[a], Takuya Suyama[a], Junko Yamashita[a], Yukiko Iguchi[a], Gang Yu[a], and Tatsuo Oshikawa[c]

[a] Graduate School of Science and Technology, Shizuoka University, Hamamatsu 432-8561, Japan

[b] Department of Internal Medicine, Hamamatsu University School of Medicine, Hamamatsu 431-3192, Japan

[c] Department of Materials Chemistry, Numazu National College of Technology, Numazu 410-8501, Japan

e-mail: tcmyama@ipc.shizuoka.ac.jp

Abstract: Diastereo isomeric *erythro* and *threo* forms of 2,3-epoxy-1-phenylphospholane 1-oxides were synthesized from *threo* and *erythro* forms of 2-bromo-3-hydroxy-1-phenylphospholane 1-oxides being prepared from 1-phenyl-2-phospholene 1-oxide. Alternatively, the epoxides were also prepared by the epoxidation of the 2-phospholene with peroxides such as sodium peroxide and hydrogen peroxide. The reactivity and regioselectivity for the reaction of *erythro* and *threo* forms of the 2,3-epoxides with nucleophiles were investigated by using amines, and the reaction afforded 2-amino-3-hydroxy-1-phenylphospholane 1-oxides, which correspond to phospha sugar *N*-glycosides. 2,3-Dibromo-3-methyl-1-phenylphospholane 1-oxides were first prepared from 3-methyl-1-phenyl-2-phospholene 1-oxide. The prepared phospholanes or phospha sugars were biologically qualified by MTT (3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide) *in vitro* method to find that some of these phosphorus heterocycles or phospha sugars have quite efficient anti-cancer activity for leukemia cells in manners of (i) wide spectra, (ii) high activities, and (iii) high specificities.

Keywords: Heterocyclic compounds, Epoxyphospholane, Tumors, Leukemia cells, MTT method

Introduction

Phospha sugars have a phosphorus atom in place of the ring oxygen atom of normal sugars, and are classified into the category of pseudo sugars. Many nucleoside

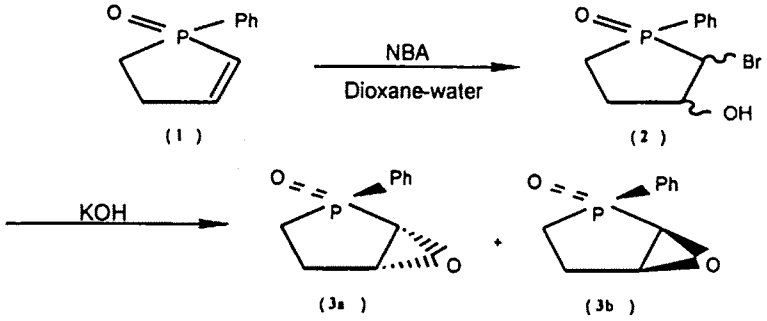
derivatives of sugars, e.g., AZT (1) and Ribavirin (2), are prepared and used as antivirus agents (1,2). In addition to the nucleoside of normal sugars, sugar modified nucleoside derivatives of pseudo sugars or hetero sugars such as aza- (3), carba- (4), and thia-sugars (5) have been synthesized and reported.

Substitution reaction of the bromo group of 2-bromophospholane derivatives, which corresponds to 2-bromo-2-deoxyphospha sugar derivatives, with amine nucleophiles gave N-glycosides of phospha sugar derivatives. Further, nucleic acid bases such as uracil were formed by the cyclization reaction of acrylamide intermediates to prepare phospha sugar nucleosides (6). We are continuously searching biologically active phosphorus heterocycles, phospha sugars and phospholanes, for a couple of ten years.

In this paper, we will deal with the successful preparation of epoxy- and bromophospholane derivatives from 1-phenyl-2- phospholene 1-oxide. The evaluation of the biological activity for leukemia cells by using 3-(4,5-Dimethyl-2-thiazolyl)-2,5- diphenyltetrazolium bromide (MTT) *in vitro* methods for these phosphorus heterocycles or phospha sugars is first reported in this paper.

Results and Discussion

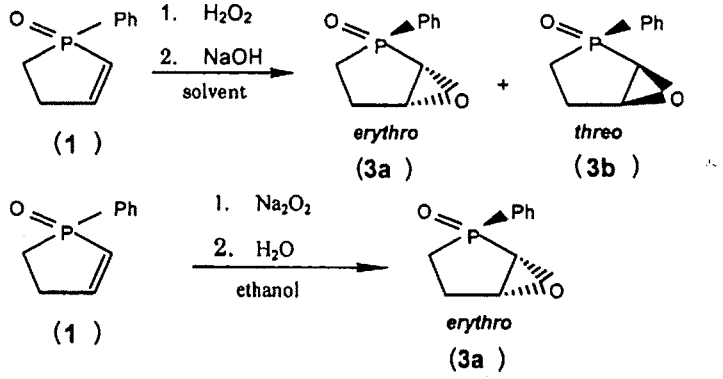
The reaction of 1-phenyl-2-phospholene 1-oxide **1** with bromine (7) in aqueous media followed by treatment with 0.5 *N* KOH aq. solution gave 2,3-epoxy-1-phenylphospholane 1-oxide **3** via bromohydrin **2**. Diastereomers of *erythro* and *threo* epoxides **3a** and **3b**, respectively, were isolated in a ratio of 5:3 (Scheme 1) by column chromatography on silica gel and the each structure was determined by ^{1}H NMR spectra.



Scheme 1: Epoxyphospholanes **3a** and **3b** from 2-phospholene **1** via bromohydrin **2**.

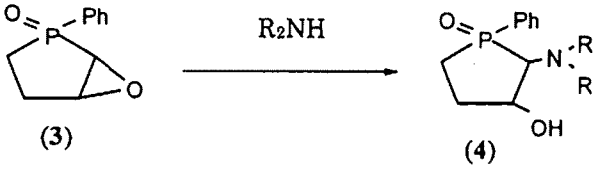
Epoxidation of **1** with sodium peroxide afforded only *erythro* **3a** under limited reaction condition (8), however, the epoxidation of **1** with one of the most popular oxidizing reagent, hydrogen peroxide, produced *erythro* **3a** and *threo* **3b** in a variable

ratio depending on the conditions (Scheme 2). On the other hand, the epoxidation with *m*-CPBA was not successful at all, because 2-phospholene 1-oxide **1** has a strong electron withdrawing phosphoryl group at the adjacent to the C=C bond, and then the C=C bond becomes electron deficient.



Scheme 2. Epoxidation of 2-phospholene **1** with peroxides to give **3**.

2,3-Epoxyphospholanes of *erythro* **3a** and *threo* **3b** were allowed to react with amines, e.g., ammonia, diethylamine, and diisopropylamine, to give 2-amino-3-hydroxy-1-phenylphospholane 1-oxide **4**, which corresponds to *N*-glycosides of phospha sugar derivatives (Scheme 3 and Table 1).



Scheme 3. Nucleophilic substitution reaction of the epoxide **3** with amines.

From the results shown in Table 1, the nucleophilic substitution reaction of 2,3-epoxyphospholanes **3a** and **3b** with amines occurred at the C-1 position and the difference of reactivity between *threo* and *erythro* diastereomers of epoxides **3a** and **3b**, respectively, was large. The substitution reaction of epoxides with amines suffers from substituent effects of amines, especially

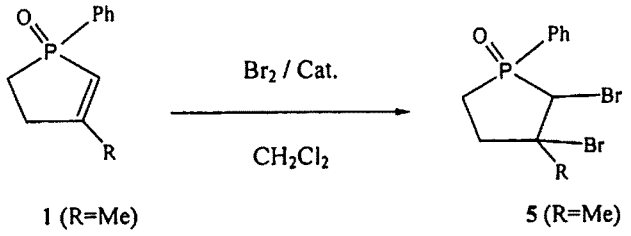
Table 1. Nucleophilic substitution reaction of the epoxide **3a** (*erythro*) and **3b** (*threo*) with

Entry	Starting material: Epoxide Diastereomer	Amine	Reaction Condition: Solv.	Temp.	Time	R	R'	Product: Compound No.	Yield (%)	Structure
1	erythro	NH_3	water	r.t.	2days	H	H	4a$_1$	20	
2	erythro	Et_2NH	CH_3OH	40°C	1week	Et	Et	4a$_2$	trace	
3	erythro	i-PrNH_2	CH_3OH	40°C	1week	Pr	Pr	4a$_3$	N.R.[a]	
4	threo	NH_3	water	r.t.	2days	H	H	4b$_1$	quant	
5	threo	Et_2NH	CH_3OH	40°C	2days	Et	Et	4b$_2$	80	
6	threo	i-PrNH_2	CH_3OH	40°C	2days	Pr	H	4b$_3$	96	
7	threo	t-BuNH_2	CH_3OH	40°C	4days	Bu	H	4b$_4$	71	
8	threo	i-Pr$_2$NH	EtOH	80°C	2days	Pr	Pr	4b$_5$	13	

a) N.R.: No reaction.

reactivity of *erythro* epoxide **3a** is very low by the steric hindrance of the phenyl group of phospholane and the alkyl group of amines. Thus, the smaller amine nucleophile, i.e., ammonia, reacts with 2,3-epoxyphospholane **3** even at room temperature, however, the larger nucleophile, i.e., diisopropylamine, is much less reactive than ammonia with the epoxide **3**.

Addition reaction of bromine with 3-methyl-1-phenyl-2-phospholene 1-oxide **1** (R=Me) with bromine in dichloromethane in the presence of catalyst, i.e., manganese dioxide, gave 2,3-dibromo-3-methyl-1-phenylphospholane 1-oxide **5** (R=Me).



1 (R=Me) 5 (R=Me)

Scheme 4. Preparation of 2,3-dibromophospholane derivative **5** (R=Me).

The prepared phospholane or phospha sugar derivatives were bio-assayed by MTT *in vitro* method for leukemia cells of K562 and U937 cell lines for the first time. Some of the results of the *in vitro* bio-assay for leukemia cell of K562 cell line with the bromohydrin **2**, epoxide **3**, and dibromide **5** of phospholanes are shown in Figures 1-3.

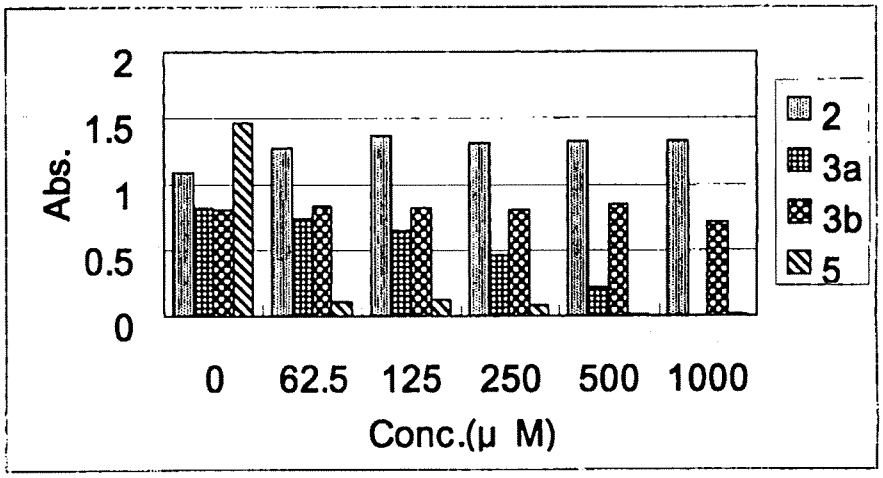


Figure 1. MTT *in vitro* evaluation results for K562 leukemia cell by bromohydrin **2**, epoxide **3**, and dibromide **5**.