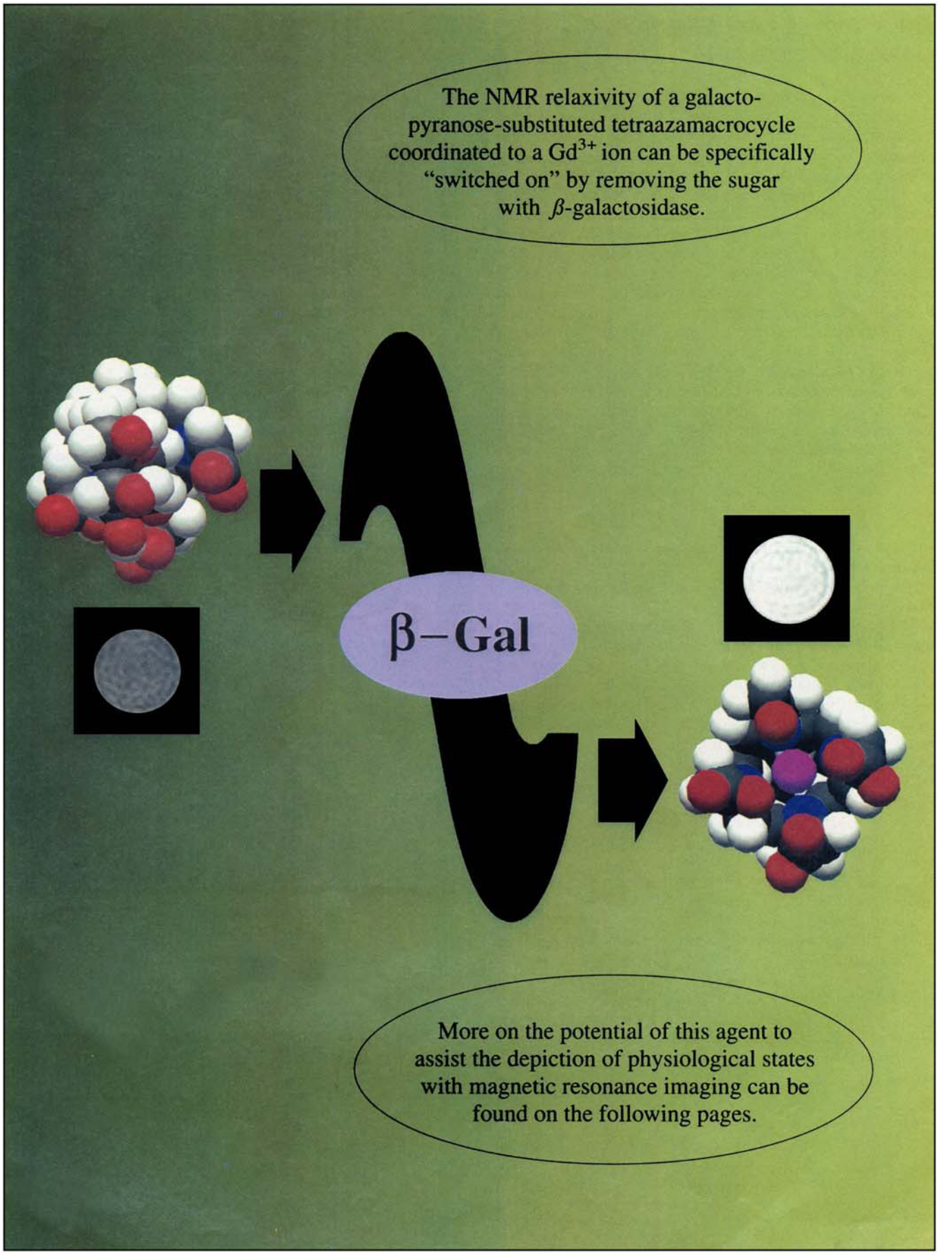

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A "Smart" Magnetic Resonance Imaging Agent That Reports on Specific Enzymatic Activity

Rex A. Moats, Scott E. Fraser, and Thomas J. Meade*

Modern optical microscopy techniques combined with fluorescent indicator compounds have revolutionized studies of biological structure, function, and development by allowing the physiology of intact cells and tissues to be assayed.[1] For example, fluorescent dyes are often injected into cells of transparent embryos in order to follow the fate of their lineal descendants. Fluorescent dyes that change their intensity or wavelength upon binding Ca^{2+} have afforded insights into cell physiology. Further, fluorogenic substrate dyes can be used to follow the activation of a specific gene by fusing the β-galactosidase gene with the gene of interest's regulatory domain. However, optical techniques, ranging from video-microscopy to laser scanning confocal microscopy, work best in the outermost 100 μm of biological tissue because of light scattering and uncorrected optical aberrations.[2]

Magnetic resonance imaging (MRI) of biological structures offers an alternative to light microscopy that can circumvent these limitations, and recent analyses demonstrate the feasibility of true three-dimensional MR imaging at cellular resolution (about 10 μm).[3] In order to exploit the power of MRI for biological studies, contrast agents that are analogous to fluorescent indicator dyes used in light microscopy must be developed. Here, we describe the first member of an innovative new class of enzymatically responsive or "smart" MRI contrast agents that has been designed to report on the metabolic state of cells and organs and transmit this information in the form of an acquired MRI image.

MRI typically imposes one or more magnetic field gradients upon a specimen while exciting nuclear spins with RF pulses. After collection of data with a variety of field gradients, computational processing yields a one-, two-, or three-dimensional image of the specimen. The image is based upon the NMR signal from the protons of water in a given volume element. Ultimately, the signal intensity arising from any volume element (voxel) of the 3D image is a function of the water concentration and relaxation times (T_1 and T_2). Local variations in these three parameters provide the vivid contrast seen in in vivo images obtained by MRI. MRI contrast agents can enhance the intrinsic differences in the T_1 (spin–lattice) and T_2 (spin–spin) relaxation rates. T_1 agents accelerate the T_1 relaxation rate, increasing the signal from nearby water protons and making the voxel appear "brighter" in the resulting image. This decrease in T_1 is mediated, in part, by the direct interaction of water molecules with the unpaired electrons of a paramagnetic metal ion.[4, 5]

The lanthanide ion Gd^{3+} is by far the most frequently chosen metal atom for MRI contrast agents, because it has a very high magnetic moment ($\mu^2 = 63\ \mu_B^2$), and a symmetric electronic ground state S^8. The Gd^{3+} aqua ion is quite toxic and must be bound to a chelating ligand in order to reduce toxicity. Typically, eight of the nine available Gd^{3+} coordination sites are occupied by the chelating ligand, leaving one site available for inner sphere coordination of a water molecule.[6] When the number of coordinated water molecules (q) is equal to 1, half the measured relaxivity of the agent is derived from second coordination sphere water molecules. The remaining 50% comes from water molecules that are directly bound to the Gd^{3+} ion, but are rapidly exchanging.

The inner sphere mechanism for T_1 relaxation phenomena suggested a means to create a contrast agent with two distinct relaxivities, weak and strong. By blocking the one remaining open coordination site, water protons would be excluded from the inner sphere and the effect of the Gd^{3+} ion on the T_1 would be diminished.[7, 8] This can be accomplished by strategically modifying the chelate ligand with a coordinating group that sterically blocks the remaining inner sphere site. For the agent described here, this blocking group is designed to be cleaved by an enzyme. The T_1 of nearby water molecules can therefore be increased by the removal of the blocking group.

The first of this new family of MRI contrast agents, (4,7,10-tri(acetic acid)-1-(2-β-galactopyranosylethoxy)-1,4,7,10-tetraazacyclododecane)gadolinium (EGad), positions a galactopyranose residue at the ninth coordination site of the Gd^{3+} ion (Figure 1).[9] Exposure of EGad to the common marker enzyme

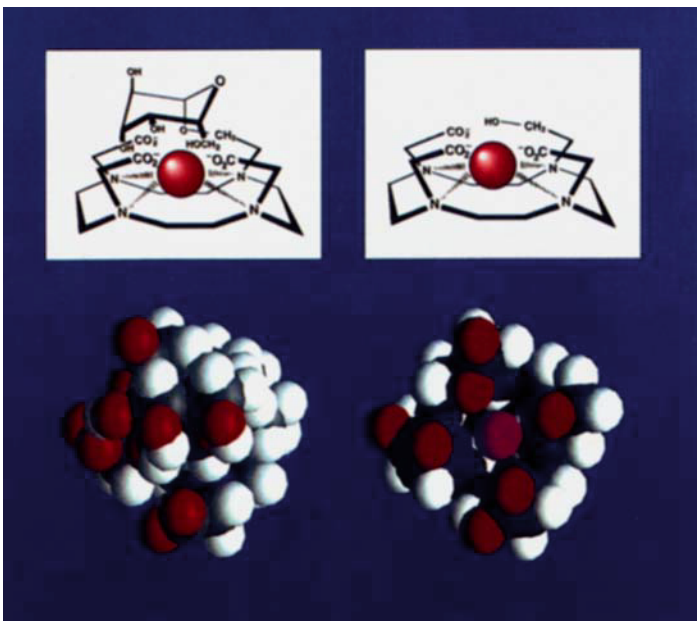

Figure 1. The MRI contrast agent with distinct weak and strong relaxivity states. Top: Structure diagram representing the attachment of the galactopyranose ring to the tetraazamacrocycle (left) and with the galactopyranose unit removed (right). Bottom: Molecular modeling illustrations of the complex with (left) and without (right) the galactopyranose side arm (the Gd^{3+} ion is depicted in magenta).

β-galactosidase (β-gal) removes the galactopyranose from the chelator and causes an irreversible transition from a weak to a strong relaxivity state. X-ray crystallographic analysis, mass spectrometry, and NMR spectroscopy confirmed the configuration and structure of the intermediates and the final product.

The ability of β-gal to remove the galactopyranose blocking group from EGad was examined by HPLC (Figure 2) by using the distinct retention times of EGad and Gad (EGad without the galactopyranose residue). When EGad was incubated with native β-gal, a peak with an elution time of 15 minutes appeared that corresponds to Gad. However, no change in the chromatogram could be detected after exposure to heat-inactivated

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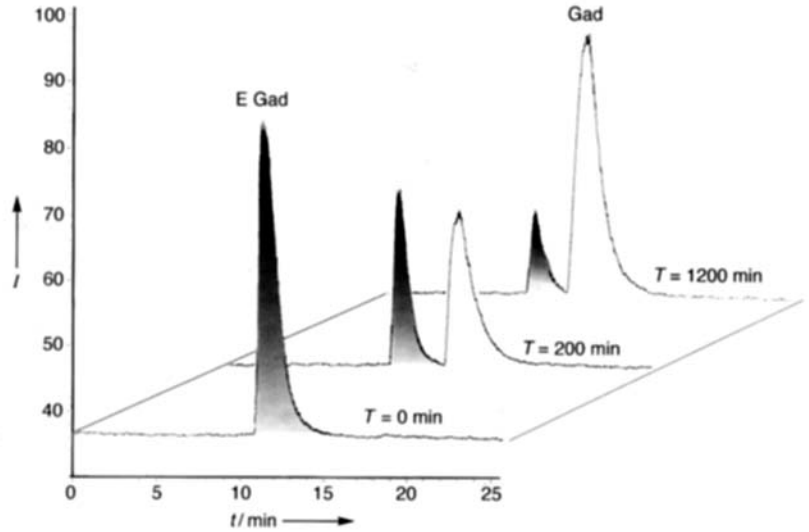

Figure 2. HPLC traces of the time dependent cleavage of the galactopyranose residue from the EGad complex by the enzyme β-gal. The Gd complex (2 mM) was incubated with β-gal (10 μM) at pH 7.3 and 37 °C in 25 mM phosphate buffer. The concentrations used approximate those required to generate MR image enhancement in vivo.

β-gal or α-galactosidase (used as negative control experiments). Thus, the HPLC experiments confirm the enzymatic processing of EGad by the appropriate, active enzyme.

An assessment of the ability of the galactopyranose residue to modulate the water exchange rate of EGad, we measured the fluorescence spectra of the terbium derivative (EGad–Tb) in water/deuterium oxide mixtures (λ_{em} 545 nm).[8, 10, 11] The fluorescence of the terbium complex is quenched by H_2O, but not by D_2O because the excited state of the terbium is strongly coupled to the OH oscillator but not the OD oscillator. Therefore, the lifetime of the fluorescence signal is longer in D_2O than in H_2O. This value was used to determine the number of water molecules (q) that are in fast exchange with the complex.[12] The q values for the terbium complexes of EGad and Gad were determined to be 0.7 and 1.2, respectively, which confirmed a significant change (about 40%) in the number of inner sphere water molecules.

The effect of the enzymatic cleavage of the galactopyranose residue from EGad on the T_1 of the complex, predicted to decrease by the spectrofluorimetry experiments, was assessed with NMR spectroscopy (Figure 3).[13] A 20% decrease in the measured T_1 values of a solution of the agent in the presence of β-galactosidase (Figure 3) is consistent with the change in measured hydration number q obtained from fluorescence measurements. Two different concentrations of β-gal resulted in identical and significant decreases in the solution T_1. Control reactions in which EGad was combined with heat-inactivated enzyme showed no decrease; in fact, the T_1 appeared to increase slightly. These results indicate that the decrease in T_1 of EGad is due to the enzymatic cleavage of the complex.

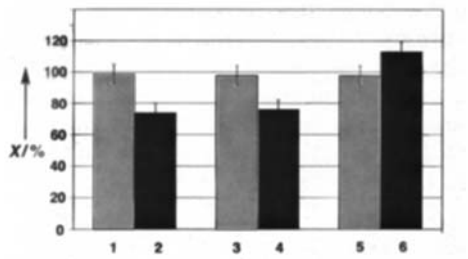

Figure 3. Change in T_1 observed upon β-gal-catalyzed cleavage of the galactopyranose residue (n = 3). The first column of each set (columns 1, 3, and 5) represent T_1 of the EGad and β-gal immediately after mixing. The second column of each set represents T_1 of the solution after incubation in the presence of β-galactosidase. Each column is reported as a ratio (X) to a control containing EGad only (that is, T_1(sample)/T_1(EGad without β-gal)). Columns 1 and 2: 2.0 mM Gd complex + 1.7 μM β-gal. Columns 3 and 4: 2.0 mM Gd + 5.1 μM β-gal. Columns 5 and 6: 2 mM Gd complex + 5.1 μM heat-inactivated β-gal.

MR images were obtained with a standard inversion–recovery sequence; the change in T_1 generated by enzymatic conversion of EGad to Gad could be visualized in an MR image (Figure 4). EGad was placed in 1.5 mm capillary tubes in the presence and absence of β-galactosidase. The images displayed in Figure 4 reveal that the T_1-mediated contrast of EGad was altered by the action of β-gal, yielding the expected image enhancement.

The successful synthesis and testing of EGad demonstrates the feasibility of using responsive MRI contrast agents to enhance images of physiological states of cells or organs deliberately. The distinct weak and strong relaxivity states of this agent offers the promise of direct, three-dimensional visualization of gene expression by MRI. Given the widespread use of β-gal as a marker enzyme in studies on mice, this new class of agent offers the immediate promise of following gene expression patterns in normal and genetically perturbed animal models.

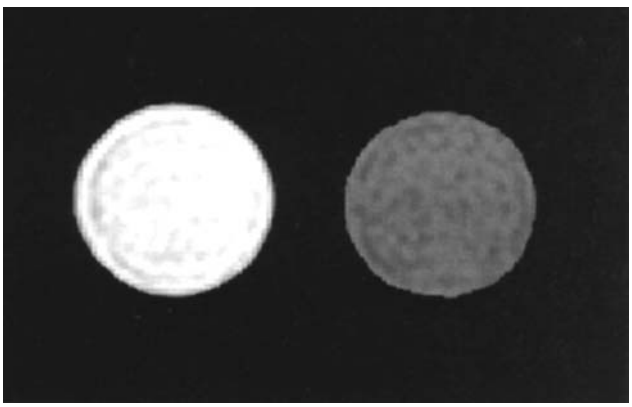

Figure 4. MRI contrast enhancement as a result of enzymatic cleavage of EGad. Images (256 × 256 × 3) of a solution of EGad in the presence (left) and absence (right) of the enzyme β-gal (2.0 mM Gd complex and 5.1 μM β-gal) were obtained horizontally through a 1.5 mm capillary tubes on a modified Bruker AMX spectrometer with a standard inversion–recovery imaging sequence and are displayed as raw data. The inversion delay was set to the average of the T_1 values (0.280 sec) and the interscan delay is 0.2 sec.

Although the issue of delivery of MRI agents to specific cell types by means other than microinjection is still under investigation,[14] the agent described here represents the first step in the evolution of a new class of image enhancement substances, and it is currently being tested in vivo. Extensions of this family of MRI contrast agents to other physiological or neuronal activation processes will permit biological issues presently inaccessible by light microscopy techniques to be investigated by MR imaging.

Experimental Section

Synthesis of 2,3,4,6-tetraacetyl-1-(2-bromoethoxy)-β-galactose: 2-Bromoethan-1-ol was treated with 2,3,4,6-tetraacetyl-1-α-bromogalactose to produce a mixture of

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α and β anomers (10/90) of 2,3,4,6-aceto-1(2-bromoethoxy)-β-galactose in 68% yield (8.3 g). The purified β anomer could be obtained by flash chromatography. Stereochemical assignments were made by an X-ray crystal structure of the β anomer.

2,3,4,6-tetraacetyl-1-(2-bromoethoxy)-β-galactose was treated with cyclene to produce the monosubstituted product. The acetate protecting groups were cleaved, and the three carboxylic acid substituents were added by reaction with bromoacetic acid at pH 10.5. The product 4,7,10-tri(acetic acid)-1-(2-β-galactopyranosylethoxy)-1,4,7,10-tetraazacyclododecane was isolated by anion exchange fast performance liquid chromatography (FPLC, detection at 218 nm) in 37% overall yield. Gd^{3+} or Tb^{3+} ions were incorporated into complexes, and these (EGad-Gd and EGad-Tb) were purified by repeated collections on a reverse phase HPLC analytic C_{18} column with water/acetonitrile (0–10% gradient) as the eluent (fluorescence detection $\lambda_{ex} = 274$ nm and $\lambda_{em} = 315$ nm) in 70% yield. The high resolution mass spectrum of the isolated solid provided a parent molecular ion $(M+Na)^+$, which exhibited the correct exact mass and the predicted isotope ratios.

X-ray data for $C_{16}H_{23}O_{10}Br$: CAD-4 diffractometer; monoclinic, colorless plates, space group $P2_1$ (no. 4); a total of 5603 reflections measured, 2981 used for refinement.

Fluorescence experiments [5,6] with EGad: The decay rate (inversely proportional to the lifetime) of the emission peak at $\lambda_{em} = 545$ nm ($\lambda_{ex} = 460$ nm) was measured with a Hitachi f-4500 fluorescence spectrophotometer (2 s delay, 64 scans) in H_2O, 50/50 H_2O/D_2O, and D_2O. An exponential curve fit (DeltaGraph 3, Delta Point Inc., Monterey, CA) was used to determine the decay rates. The slope of the decay rates versus D_2O concentration was compared to the literature value of a slope of $0.239/q$ to obtain q.

In three identical inversion–recovery (IR-NMR) high resolution experiments (Bruker AMX 500, 26 °C) EGad(2 mM) was incubated with two different concentrations of β-gal (1.7 μM and 5.1 μM) heat-inactivated β-gal (10 min at 80 °; 5.1 μM), and EGad alone (2 mM) in phosphate buffer (25 mM, pH 7.3) at 37 °C. Minimal enzyme concentrations were used to reduce potential interactions between the contrast agent and the enzyme. The solutions in a 40 μL round-bottomed NMR tube insert (Wilmad glass) were placed into a 5 mm NMR tube containing CD_3Cl. T_1 measurements were made immediately following mixing and after complete cleavage of the galactopyranose (>95% after incubation for 7 days). The data was processed with the program Felix (BIOSYM/Molecular Simulations, San Diego, CA), and the peak heights were fitted to an exponentially rising curve in order to obtain T_1 (regression: $R>0.999$).

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[1] R. Y. Tsien, *ACS Symp. Ser.* **1993**, *538*, 130–146.
[2] H. E. Keller in *Handbook of Biological Confocal Microscopy*, 2nd ed. (Ed.: J. B. Pawley), Plenum, New York, **1995**, p. 111.
[3] R. W. Bowtell, J. C. Sharp, P. Mansfield, E. W. Hsu, N. Aiken, A. Horsman, *Mag. Res. in Med.* **1995**, *33*, 790–794; C. T. Rofe, J. Vannoort, P. J. Back, P. T. Callaghan, *J. Mag. Res. Ser. B.* **1995**, *108*, 125–136; Z. P. Liang, P. C. Lauterbur, *IEEE Trans. Med. Imaging* **1994**, *13*, 677–686; R. E. Jacobs, S. E. Fraser, *Science* **1994**, *263*, 681; A. F. Mellin, G. P. Cofer, B. R. Smith, S. A. Suddarth, L. W. Hedlund, G. A. Johnson, *Mag. Reson. Med.* **1994**, *32*, 199–205; W. Kuhn, *Angew. Chem.* **1990**, 29, 1–19.
[4] I. Bertini, C. Luchinat, *NMR of Paramagnetic Molecules in Biological Systems*, Benjamin/Cummings, **1986**.
[5] S. H. Koenig, K. E. Kellar, *Magn. Res. Med.* **1995**, *34*, 227–233.
[6] V. Alexander, *Chem. Rev.* **1995**, *95*, 273–242; K. Kumar, C. A. Chang, M. F. Tweedle, *Inorg. Chem.* **1993**, *32*, 587–593.
[7] L. S. Szczepaniak, A. Sargeson, I. I. Creasei, R. J. Geue, M. F. Tweedle, R. G. Bryant, *Bioconjuagate* **1992**, *3*, 27–31.
[8] K. Kumar, M. F. Tweedle, *Pure Appl. Chem.* **1993**, *65*, 515–520.
[9] S. I. Kang, R. S. Ranganathan, J. E. Emswiler, K. Kumar, J. Z. Gougoutas, M. F. Malley, M. F. Tweedle, *Inorg. Chem.* **1993**, *32*, 2912–2918; S. Aime, M. Botta, G. Ermondi, *ibid.* **1992**, *31*, 4291–4299.
[10] M. Li, P. R. Selvin, *J. Am. Chem. Soc.* **1995**, *117*, 8132–8138.
[11] W. D. Horrocks, D. R. Sudnick *J. Am. Chem. Soc.* **1979**, *101*, 334–339.
[12] X. Zhang, C. A. Chang, H. G. Brittain, J. M. Garrison, J. M. Telser, M. F. Tweedle, *Inorg. Chem.* **1992**, *31*, 5597–5600.
[13] The molar quantity related to these T_1 values is the relaxivity R. R values at 500 MHz were determined for EGad (1800 $mM s^{-1}$) and Gad (2400 $mM s^{-1}$mM s^{-1}) and compared to that of a related species, DO3a (2700 $mM s^{-1}$).
[14] J. F. Kayyem, R. M. Kumar, S. E. Fraser, T. J. Meade, *Chem. and Bio.* **1995**, *2*, 615–620.

Glycylglycine Rotaxanes— The Hydrogen Bond Directed Assembly of Synthetic Peptide Rotaxanes

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Although DNA displays a range of hydrogen bond assembled, topologically distinct architectures,[1–4] only recently have knotted,[5–7] catenated,[5,6,8,9] and rotaxanated[5,6,10–12] substructures been identified in polypeptides and proteins. Unnatural DNA knots and catenanes of tremendous complexity can be synthesized[13–15] and are seen as promising forerunners to new types of drug delivery systems, nanoscale mechanical devices, and even "biochips".[16,17] Here we report that glycylglycine derivatives can be used as templates for the formation of benzylic amide macrocycles through a five-molecule, hydrogen bond directed "clipping" strategy to give peptido[2]rotaxanes in yields as high as 62%. The four intercomponent hydrogen bonds responsible for rotaxanation "live on" in nonpolar solvents such as chloroform and, in the case of pyridine-2,6-dicarbamidobenzyl macrocycles, in the solid state and in polar solvents such as dimethylsulfoxide and dimethylsulfoxide–water mixtures. In the latter, the macrocycle forms an impenetrable molecular sheath over part of the peptide backbone making it inaccessible to external reagents as small as D_2O.

Benzylic amide [2]catenanes such as **1** are a structurally diverse family of catenanes most conveniently prepared by the eight-molecule condensation of aromatic 1,3-dicarboxylic acid dichlorides and benzylic diamines in nonpolar solvents.[18,19] If the reactions are carried out in the presence of a suitably stoppered benzylic 1,3-diamide "thread" such as **2** [2]rotaxanes such as **3** are also formed.[20] In both cases the mechanism for the formation of the topologically complex products appears to be primarily the directed assembly of the macrocycle around two transoid amide bonds (**4**, Scheme 1).[21] Divergent hydrogen bonding sites in a similar spatial arrangement occur in adjacent amino acid residues in peptide chains, and therefore it seemed possible that these could also carry the correct structural information (**5** and **6**) to template the cyclization of benzylic amide macrocycles to give rotaxanes (Scheme 2).

To test this hypothesis the simplest dipeptide, glycylglycine, was incorporated into a suitable thread. The commercially available glycylglycine ethyl ester was N-acylated with diphenylacetyl chloride (Et_3N, THF, 90%) and then transesterified[22] at the carboxylate terminus with 2,2-diphenylethanol ($(Bu_2SnCl)_2O$, toluene, Δ, 90%). Equimolar quantities of isophthaloyl dichloride and *p*-xylylene diamine were slowly added to a solution of the dipeptide thread in anhydrous $CHCl_3$ (Scheme 2a). After five equivalents had been added, the thread was no longer consumed. Filtration and washing with acid and base left only three components in solution. These were separated by flash chromatography and identified in order of elution as the peptido[2]rotaxane **8** (62% yield), the unrotaxanated thread **7**, and [2]catenane **1**.

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