Expedited Articles

A Modular Lymphographic Magnetic Resonance Imaging Contrast Agent: Contrast Enhancement with DNA Transfection Potential

Erik R. Wisner,† Kimberly L. Aho-Sharon, Michael J. Bennett, Sharron G. Penn, Carlito B. Lebrilla, and Michael H. Nantz*

Departments of Chemistry and Radiology, University of California, Davis, California 95616

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A gadolinium-chelated liposomal contrast agent has been prepared, and magnetic resonance imaging (MRI) efficacy has been examined by indirect magnetic resonance lymphography. A lipidic *N,N′*-dimethylethylenediamine derivative (**4**) containing a 10,12-diyne-diacyl domain was treated with DTPA anhydride followed by $GdCl_3$ complexation. The complex was confirmed using MALDI spectrometry. An equimolar mixture of the Gd−chelate lipid and a commercially available diyne-PE was formulated as a liposome suspension and irradiated with UV light prior to imaging experiments. Subcutaneous injection of the liposomal gadolinium agent and subsequent MRI of rabbit axillary and popliteal lymph nodes revealed significant contrast enhancement up to 4 h postinjection. To explore the possibility of imaging a DNA transfection event, the gadolinium contrast mixture was formulated with the cationic transfection lipid DOTAP and complexed with the reporter gene encoding luciferase. DNA transfection studies on the NIH3T3 cell line confirmed the transfection activity of the dual-purpose contrast agent and exemplified the potential toward development of an imaging and DNA delivery vehicle.

Since the advent of magnetic resonance imaging (MRI) for medical diagnostics, there has been continued interest in the design and synthesis of paramagnetic complexes for contrast enhancement.[1] Among the MRI contrast agents that have been developed, gadolinium-(III) complexes have been shown to be highly effective in their imaging properties.[2] However, the clinical utility of a contrast agent depends not only on absolute contrast-enhancing properties but also on the pharmacokinetics that determine distribution and concentration of the contrast agent in target tissues. Indirect MR lymphography, a technique in which subcutaneously injected contrast material selectively accumulates within regional lymph nodes, has recently gained attention as an approach for diagnosing and staging cancer.[3] The most widely available gadolinium contrast agent, gadolinium diethylenetriaminepentaacetic acid (Gd-DTPA), is not used for lymphographic MRI because of its rapid distribution within the extracellular space and rapid renal clearance.[4] To overcome this limitation, one approach has been to incorporate the gadolinium into liposomal lamellae.[5,6] Incorporation of the paramagnetic species into liposomes can result in increased accumulation within the lymph nodes by intranodal macrophage uptake of particulate species. We seek to extend this approach by introducing a polymerizable lipid formulation that is capable of binding gadolinium as well as binding a polynucleotide.[7] Lipid-mediated DNA delivery as a means for gene therapy is a developing modality for the treatment of cancer and other diseases,[8] and the combination of this methodology with selective, contrast-enhanced MRI would improve its utility. Visualization of polynucleotide localization within lymph nodes by gadolinium contrast enhancement would be a rapid, noninvasive means of monitoring treatment, were the same agent also capable of simultaneously serving as the gene transfection agent. A report by Meade et al. on the cotransport of DNA and DTPA-Gd using polylysine in cell culture experimentation supports the feasibility of this approach.[9]

We report herein the synthesis of a prototypic gadolinium-chelated lipid and its modular incorporation with a commercially available DNA-binding lipid to yield a dual-purpose liposome formulation for lymphographic imaging and treatment. Preliminary imaging and DNA transfection properties are presented.

Results and Discussion

Synthesis. The synthesis of the gadolinium chelate lipid is shown in Scheme 1. Treatment of 10,12-octadecadiynoyl chloride (**2**) with 3-bromo-1,2-propanediol yields bisester **3**. The bromide of **3** is readily displaced by treatment with excess *N,N′*-dimethylethylenediamine at elevated temperature. Reaction of **3** using fewer equivalents of diamine or longer reaction times affords significantly lower yields of diamine **4**. Amide formation by reaction of **4** with excess DTPA-anhydride in pyridine is followed by hydrolysis of unreacted anhydride. The corresponding amide mixture[10] was reacted with Gd(III) according to the procedure by Bednarski et al.,[7a] and the resultant chelate **5** was analyzed by matrix-assisted laser desorption ionization (MALDI) spectrometry. The MALDI spectrum of **5** confirms an isotopic distribution characteristic of a gadolinium species, centered around the expected molecular weights.[11]

Magnetic Resonance Imaging. To determine the contrast properties of the gadolinium-bound lipid **5**,

* To whom correspondence should be addressed. Phone: 503 752 6357. Fax: 503 752 8995. E-mail: mhnantz@ucdavis.edu.
† Department of Radiology.
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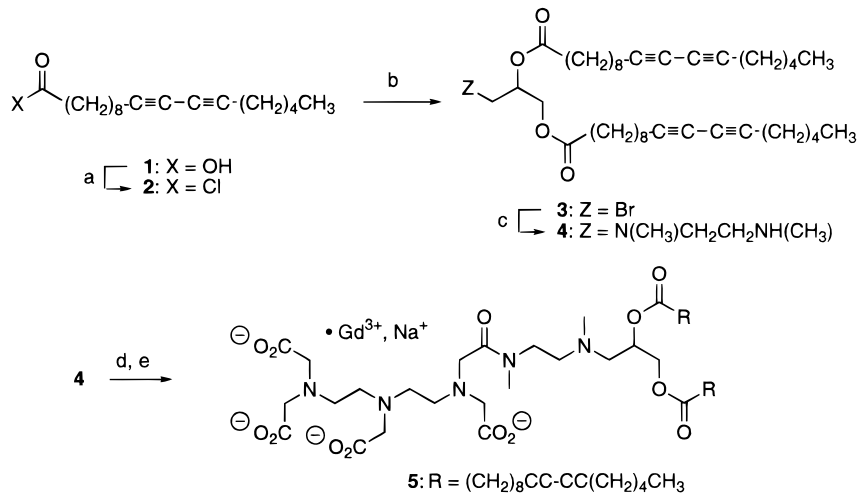
Scheme 1. Synthesis of Polymerizable Gd−Chelate Lipid[a]



[a] Reagents and conditions: (a) $(CO)_2Cl_2$, $CHCl_3$, reflux; (b) $BrCH_2CH(OH)CH_2OH$ (0.45 equiv), Et_3N (2.2 equiv), cat. DMAP, CH_2Cl_2; (c) $HN(CH_3)CH_2CH_2NH(CH_3)$ (15 equiv), DMF, 75 °C, 45 min; (d) i. DTPA-anhydride (3.5 equiv), pyridine, 50 °C, 12 h, ii. H_2O; (e) $GdCl_3 \cdot 6H_2O$, $NaOCH_3$, CH_3OH, 60 °C, 30 min.

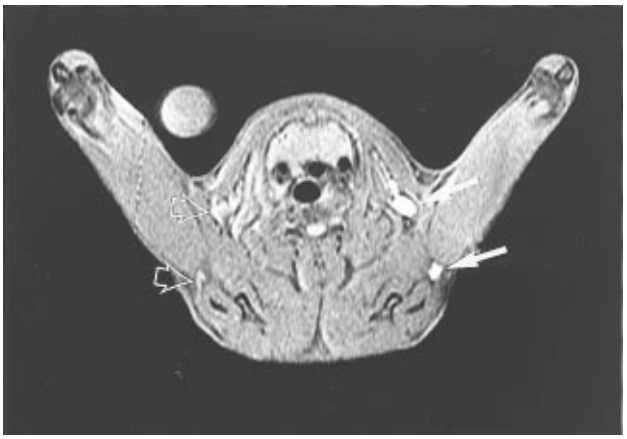


Figure 1. T_1-weighted transaxial MR image of contrast-enhanced axillary and superficial cervical lymph nodes in a normal New Zealand White rabbit (solid arrows). This image was acquired approximately 120 min after subcutaneous injection of 0.4 mL of the Gd-bound suspension (17×10^{-6} mol of Gd) into the ipsilateral metacarpus. The unenhanced contralateral nodes are poorly visualized (open arrows).

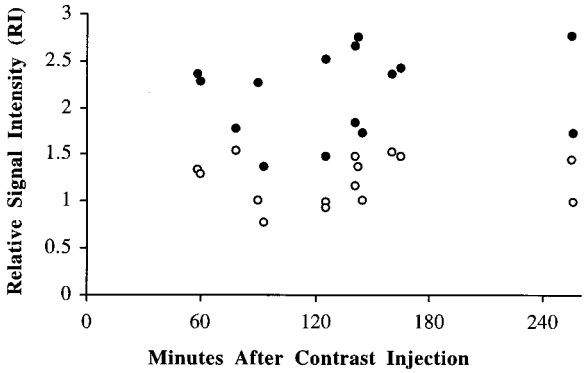


Figure 2. Relative signal intensity (RI) of lymph nodes on T_1-weighted spin−echo magnetic resonance images versus time postcontrast injection: (●) RI of all contrast-enhanced lymph nodes at each time point analyzed, (○) RI of corresponding unenhanced contralateral lymph nodes at each time point.

preliminary MRI studies were performed on three normal rabbits. The contrast material, polymerized liposome formulations containing equimolar quantities of lipid **5** and 1,2-bis(10,12-tricosadiynoyl)-*sn-glycero*-3-phosphoethanolamine (**6**),[12] serving as a polymer matrix lipid, was prepared by sonication of the aqueous lipid suspension followed by UV photolysis (0 °C, 1 h). Each animal was anesthetized and then injected subcutaneously with an empirically determined dose of contrast material at the level of the left metacarpus or metatarsus to enhance the axillary/cervical or popliteal lymph nodes, respectively. Earliest images were acquired beginning approximately 1 h after contrast administration and at 30−90-min intervals for up to 260 min postinjection. A representative image is presented in Figure 1.

Images were obtained from at least two time points for each lymph node. Using the current formulation to assess seven lymph nodes in three rabbits, relative signal intensity (RI, mean signal intensity of the node divided by the mean signal intensity of surrounding muscle tissue) increased from 1.16 in unenhanced nodes to 2.19 in contrast-enhanced nodes at peak enhancement ($p > 0.0002$, two-sample t-test) (Figure 2). This resulted in a 91.7% peak percent contrast enhancement, PCE. Peak enhancement generally occurred 60−130 min after contrast injection, and enhancement persisted up to 4 h postinjection. This result demonstrates the usefulness of the polymerized liposome construct in promoting lymph node retention of gadolinium relative to Gd-DTPA preparations.

DNA Transfection Using the Gd−Liposome Formulation. Cationic lipids have been demonstrated to facilitate intracellular uptake of DNA in a number of in vitro and in vivo studies.[13,14] Typically, a cationic liposome suspension is prepared and treated with DNA so that the resultant lipid−DNA complex (referred to as a lipoplex[15]) retains an overall positive charge. Direct application of the lipoplex to cultured cells then can result in intracellular delivery and expression of the DNA. To see the influence of gadolinium incorporation on DNA transfection, a luciferase transfection assay was conducted using the methods we have previously reported.[16] Liposomes formulated using *N*,*N*,*N*-trimethyl-*N*-(1-(2,3-dioleoyl)propyl)ammonium iodide (DOTAP[17])

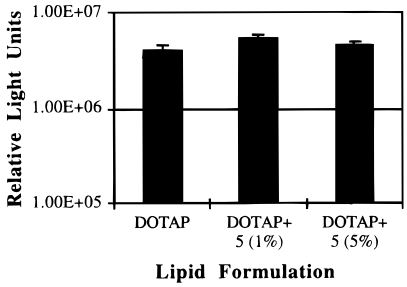


Figure 3. Comparison of lipid-mediated DNA transfection using a 2:1 molar charge ratio (DOTAP charge to DNA phosphate charge) to transfect NIH3T3 cells. DNA transfections were performed in quadruplicate, and the results are summarized in bar graph form as the mean (n = 4) and standard deviation of total luciferase light units obtained as described in the Experimental Section.

and gadolinium-chelate lipid **5** were used to bind pND-CLux, plasmid DNA encoding the firefly luciferase gene.[18] The resultant lipoplexes were used to transfect NIH3T3 murine fibroblast cells (Figure 3) at an optimal DOTAP:DNA molar charge ratio. Liposomes formulated with chelate **5** showed activity comparable to the control experiment of DOTAP. The presence of up to 5% gadolinium did not reduce the relative activity of the DOTAP lipoplex. Our results support the development of a dual-purpose contrast agent in that the selected MRI agent does not interfere with in vitro transfection. Studies are underway to extend the application to in vivo transfection of rabbit lymph nodes.

In conclusion, we have shown the possibility for development of a dual-purpose imaging and DNA delivery vehicle. The inherent modular construction of a lipoplex formulation was exploited by incorporation of a gadolinium−chelate lipid and a DNA-binding lipid. The resultant particle was shown to facilitate DNA transfection. Furthermore, a liposomal formulation of the gadolinium lipid was shown to have excellent in vivo contrast enhancement with prolonged lymph node retention.

Experimental Section

General. CH_2Cl_2 was distilled from calcium hydride immediately prior to use. MeOH was heated over Mg turnings for 12 h, and distilled prior to use. All amine reagents were distilled from CaH_2. All reactions were carried out under an atmosphere of argon. The acyl halides were prepared using a modification of the experimental reported by Clark et al.[19] Column chromatography was carried out using 230−400 mesh silica gel, slurry packed in glass columns, eluting with the solvents indicated. Yields were calculated for material judged to be homogeneous by TLC and NMR. TLC was performed on Merck Kieselgel 60 F_{254} plates, staining with a solution of phosphomolybdic acid in ethanol containing 3% concentrated H_2SO_4.

^{1}H NMR (300 MHz) and ^{13}C NMR (75 MHz) were recorded using a General Electric QE-300 spectrometer with residual undeuterated solvent (δ 7.26 for $CHCl_3$, δ 4.78 for CD_3OH) serving as the internal standard. High-resolution mass spectrometry was performed by Mass Spectrometry Service Lab, Minneapolis, MN. Melting points were determined on a Thomas-Hoover Uni-melt apparatus and are uncorrected. Ultraviolet (UV) spectra were obtained using a HP8450A UV/vis spectrophotometer. Infrared (IR) data were obtained on neat samples unless otherwise indicated using a Mattson Galaxy Series FTIR 3000 instrument.

3-Bromo-1,2-bis(10,12-octadecadiynoyl)propane (3). Oxalyl chloride (6.32 mL, 72.5 mmol) was added slowly to a solution of 10,12-octadecadiynoic acid (**1**) (10.0 g, 36.3 mmol) in chloroform (13.5 mL). The resultant reaction solution was stirred for 10 min at room temperature and then heated at reflux temperature for 20 min. All solvents were then removed by distillation to afford crude acyl chloride (**2**) as a brown oil. The crude material was dissolved in CH_2Cl_2 (5 mL) and transferred via cannula to a solution of 3-bromo-1,2-propanediol (1.32 mL, 15.1 mmol), Et_3N (4.54 mL, 32.6 mmol), and DMAP (0.181 g, 1.48 mmol) in CH_2Cl_2 (10 mL) at 0 °C. After 10 min, the reaction mixture was warmed to room temperature and stirred overnight. The reaction mixture was then diluted with CH_2Cl_2, washed with saturated $NaHCO_3$, H_2O, and saturated brine, and dried (Na_2SO_4). The solvents were removed by rotary evaporation, and the residue was chromatographed using silica gel (hexane−ethyl acetate, 9:1) to give 9.71 g (96%) of **3** as an oil: IR 2930, 2855, 2253, 2145, 1742 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 5.17 (m, 1H), 4.31 (dd, J = 11.8, 4.3 Hz, 1H), 4.18 (dd, J = 11.8, 5.6 Hz, 1H), 3.46 (m, 2H), 2.28 (m, 4H), 2.19 (m, 8H), 1.57 (m, 4H), 1.45 (m, 8H), 1.26 (m, 24H), 0.85 (t, J = 7.35 Hz, 6H); ^{13}C NMR ($CDCl_3$) δ 172.6, 172.2, 77.1, 77.0, 69.9, 65.6, 65.5, 62.9, 33.8, 31.0, 30.0, 29.3, 29.2, 29.1, 29.0, 28.9, 28.7, 28.6, 28.4, 28.3, 28.1, 24.8, 23.7, 22.2, 19.1, 18.3, 13.9; HRMS calcd for $C_{39}H_{59}BrO_4$ [M^{79}Br (49.77) + NH_4]$^+$ 688.3941, found 688.4003; calcd for $C_{39}H_{59}BrO_4$ [M^{81}Br (41.40) + NH_4]$^+$ 690.3920, found 690.3935.

3-(*N*-Methyl-*N*-(2-(*N*′-methylamino)ethyl)amino)-1,2-bis(10,12-octadecadiynoyl)propane (4). *N*,*N*′-Dimethylethylenediamine (3.57 mL, 33.5 mmol) was added to a solution of **3** (1.50 g, 2.24 mmol) in DMF (11.2 mL) at room temperature. The reaction mixture was heated to 75 °C and stirred for 45 min. The DMF was removed by distillation, and the residue was diluted with CH_2Cl_2, washed with saturated $NaHCO_3$, H_2O, and saturated brine, and dried (Na_2SO_4). The solvent was removed by rotary evaporation, and the residue was chromatographed using silica gel (CH_2Cl_2−MeOH, gradient of 99:1 to 95:5) to give 0.739 g (49%) of **4** as an oil: IR 2931, 2856, 2256, 2158, 1738 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 5.10 (m, 1H), 4.34 (dd, J = 12.0, 3.1 Hz, 1H), 4.07 (dd, J = 12.0, 6.2 Hz, 1H), 2.95 (m, 2H), 2.78 (m, 2H), 2.74 (s, 3H), 2.53 (d, J = 6.5 Hz, 2H), 2.34 (s, 3H), 2.29 (m, 4H), 2.23 (m, 8H), 1.59 (m, 4H), 1.51 (m, 8H), 1.29 (m, 24H), 0.88 (t, J = 7.1 Hz, 6H); ^{13}C NMR ($CDCl_3$) δ 173.5, 173.4, 77.0, 76.5, 69.5, 65.2, 65.1, 63.5, 57.8, 53.6, 46.6, 42.8, 34.2, 34.0, 33.2, 30.8, 29.0, 28.9, 28.8, 28.6, 28.5, 28.3, 28.2, 27.9, 24.7, 22.0, 19.0, 13.8; HRMS calcd for $C_{43}H_{70}N_2O_4$ [M + H]$^+$ 679.5413, found 679.5398.

3-(*N*-Methyl-*N*-(2-(*N*′-DTPA-amido-*N*′-methylamino)-ethyl)amino)-1,2-bis(10,12-octadecadiynoyl)propane and Gadolinium Complexation (5). DTPA-anhydride (2.34 g, 6.54 mmol) was added to a solution of **4** (1.27 g, 1.87 mmol) in pyridine (9.35 mL) at room temperature. The reaction mixture was heated to 50 °C and stirred overnight. The cooled reaction mixture was filtered to remove unreacted DTPA-anhydride, and distilled water (5 mL) was added to the filtrate. The solvents were removed by distillation under reduced pressure. The crude product was dissolved in DMSO. Any undissolved solids were removed by filtration. The DMSO was then removed in vacuo to afford 1.77 g (90%) of the DTPA conjugate as a tan solid: mp 183−184 °C; IR 3414, 2931, 2864, 2256, 2160, 1742, 1641 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 5.13 (m, 1H), 4.27 (m, 1H), 4.02 (m, 1H), 3.76 (m, 2H), 3.63 (m, 2H), 3.47 (s, 8H), 2.92 (s, 3H), 2.51 (s, 3H), 2.32 (m, 4H), 2.27 (m, 8H), 1.45 (m, 12H), 1.30 (m, 24H), 0.86 (t, J = 6.8 Hz, 6H); ^{13}C NMR (DMSO-d_6) δ 172.8, 172.7, 172.6, 76.9, 69.5, 63.5, 57.8, 54.1, 46.9, 42.7, 34.2, 34.0, 33.5, 31.7, 29.6, 29.4, 29.3, 29.1, 29.0, 27.1, 27.1, 27.0, 24.8, 22.5, 13.9; HRMS calcd for $C_{57}H_{91}N_5O_{13}$ [M + Na]$^+$ 1076.6511, found 1076.6517.

To a suspension of the DTPA conjugate (1.00 g, 0.950 mmol) in MeOH (2.38 mL) at room temperature was added $GdCl_3$·$6H_2O$ (0.353 g, 0.950 mmol) in one portion. The reaction mixture was heated to 60 °C for 30 min, and the solution pH was maintained near 7.0 by immediate and continuous monitoring and addition of $NaOCH_3$ (1.6 M in methanol) as needed. The methanol was removed by rotary evaporation, and the residue was redissolved in anhydrous methanol (ca. 5 mL). Undissolved solids were removed by filtration, and the filtrate was concentrated to afford 1.18 g of **5** as a tan solid: mp 258

°C dec; IR 3400, 2928, 2856, 2253, 2156, 1739, 1735, 1609 cm^{-1}; UV 224, 238, 254 nm; HRMS calcd for $C_{57}H_{87}GdN_5O_{13}$ 1253.5337, found 1253.5306.

Preparation of Liposomes for MRI. Sterile water (1.3 mL) was added to a vial containing **5** (51.4 mg, 0.0425 mmol) and an equimolar amount of the commercially available 1,2-bis(10,12-tricosadiynoyl)-*sn-glycero*-3-phosphoethanolamine. The suspension was vortex-mixed for 2 min and sonicated for 45 min (Laboratory Supplies Co., Inc., model G112SP1T). The liposome formulation was then transferred to a Petri dish using sterilized water (0.1 mL) and photolyzed (Hanovia Hg lamp; 254, 313, 366 nm) for 65 min at 0 °C (ice). The photolyzed preparation was stored overnight at −4 °C prior to the MRI.

Preparation of Liposomes for DNA Transfection. A chloroform solution of DOTAP (1 μmol) and a methanol solution of gadolinium complex **5** (0.01 or 0.05 μmol) were combined in a 3.7-mL sample vial. The solvents were removed via rotary vacuum evaporation, and the resulting thin lipid film was placed under vacuum overnight. Sterile water (1 mL) was added, and the thin lipid film was hydrated by briefly warming at 60 °C under argon with subsequent vortex mixing. The resultant lipid suspension was used for DNA transfection within 3 h of hydration.

Magnetic Resonance Imaging. MRI experiments were performed with a quadrature coil on a 1.5-T magnet (Signa 1.5, GE Medical Systems, Milwaukee, WI). Each rabbit was induced and maintained under anesthesia using intramuscularly injected ketamine and xylazine for imaging procedures. T_1-weighted images were obtained 60−240 min after SQ injection of the liposome suspension (0.4 mL, 17×10^{-3} mmol of Gd) into the ipsilateral metatarsus and metacarpus. Studies were performed using the following pulse sequence: T_1-weighted spin−echo (SE) TE = 11 ms/Fr, TR = 550 ms, rectangular FOV measuring 22 × 16 cm, 3-mm slice thickness with a 1.5-interslice gap, 512 × 224 matrix size, 3 excitations with chemical shift fat suppression.

DNA Transfection. NIH3T3 cells were obtained from ATCC (CRL 1658), cultured in Dulbecco's Modified Eagle's Medium with 10% calf serum and plated on standard 24-well tissue culture plates 12−24 h prior to transfection. Cells were approximately 80% confluent at the time of transfection. The growth medium was removed by aspiration, and the cells were washed once with 0.5 mL of PBS/well. The liposome−DNA complexes were formed through sequential addition of appropriate amounts of DMEM (serum-free), pNDCLux DNA, and the liposome formulation into a 2 mL Eppendorf tube to a total volume of 800 μL. Typically, 24 μL of a lipid emulsion (1 mM DOTAP) was used to complex 4 μg of DNA to yield a 2:1 DOTAP:DNA phosphate molar charge ratio. A 200-μL aliquot of the resultant transfection complex was added to each well (1 μL DNA/well, 4 wells/sample) and the cells were incubated for 4 h at 37 °C. At this time, 500 μL of growth medium and 10% calf serum was added per well, and the cells were cultured for approximately 48 h prior to lysis and analysis.

Relative luciferase activity was determined by using the Enhanced Luciferase Assay Kit and a Monolight 2010 luminometer (both from Analytical Luminescence Laboratories, Ann Arbor, MI). This was accomplished by directly applying 233.3 μL of concentrated luciferase lysis buffer (final concentration 0.1 M potassium phosphate, pH 7.8, 1% Triton X-100, 1 mM DTT, 2 mM EDTA) to each well and placing the cells on ice for 15 min. Luciferase light emissions from 31 μL of the lysate were measured over a 10-s period, and results are expressed as a function of an assumed total lysate volume of 933.3 μL. Activity is measured as relative light units, which are a function of assay conditions, luciferase concentration, luminometer photomultiplier tube sensitivity, and background. Under the conditions described above, relative light units are related to luciferase protein mass by the equation fg of luciferase = (RLU/48.6) − 824.

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Supporting Information Available: Analytical data and procedures for compounds **3**−**5** (2 pages). Ordering information is given on any current masthead page.

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