JOC Article

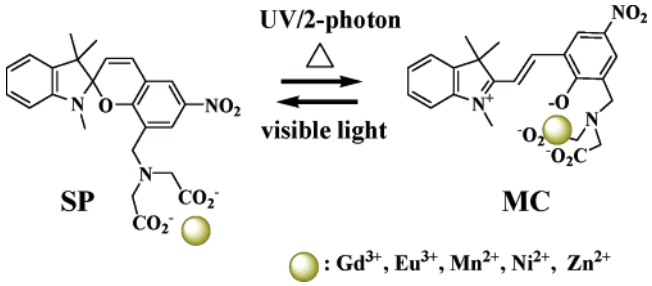
Optically Switchable Chelates: Optical Control and Sensing of Metal Ions

Tomoyo Sakata,† David K. Jackson, Shu Mao, and Gerard Marriott*

Department of Physiology, University of Wisconsin, 1300 University Avenue, Madison, Wisconsin 53706

marriott@physiology.wisc.edu

Received September 20, 2007



This study introduces new concepts in the design, synthesis, and in vitro and in vivo characterization, manipulation, and imaging of organic chelates whose association with metal ions is rapidly and reversibly controlled by using light. Di- and tricarboxylic group bearing photochromes, nitrobenzospiropyran (nitroBIPS), undergo rapid and reversible, optically driven transitions between their spiro (SP) and fluorescent merocyanine (MC) states. The MC state of nitroBIPS-8-DA binds tightly to various metal ions resulting in specific shifts in absorption and fluorescence, and the dissociation constant for its Gadolinium complex in water is measured at ~5 μM. The metal-bound MC state is converted to the weaker-binding SP state with use of 543 nm light, while the SP to MC transition is complete with use of 365 or 720 nm (2-photon) light within several microseconds. Fluorescence imaging of the MC state of nitroBIPS-8-TriA was used to quantify the rate and efficiency of optical switching and to provide a real-time readout of the state of the optically switchable chelate within living cells.

Spatio-temporal control of cell Ca^{2+} is a key feature of signaling pathways that regulate transcription, proliferation, and motility.[1] Through the development and optimization of optical probes for calcium[2] it is now understood that spatio-temporal control of Ca^{2+} serves as both an elemental molecular event in cell signaling as well as a vehicle to transmit sensory events throughout the cell.[2−6] Further information on the role of calcium transients in cell signaling has emerged from studies using caged calcium ion probes, which undergo UV-driven reactions that release Ca^{2+}. For example, UV-irradiation of cell loaded Nitr-5 and caged-EGTA can be used to generate spatially defined transients of Ca^{2+} on a millisecond time scale.[7−10] However, photochemical transitions using 2-nitrobenzyl groups have complicated excited state chemistry[11,12] with low quantum yields (~0.1).[12] Cell based investigations with 2-nitrobenzyl caged groups therefore require control studies to prove that any response of a cell to UV-irradiation results from perturbation of Ca^{2+} rather than an effect associated with UV light or the photoproducts. Furthermore, the buildup of photoproducts and their secondary reactions[11] restricts the number of uncaging events that can be performed within a single cell. We argue that a more suitable probe to control divalent metal ions in cells

* Address correspondence to this author.
†Current address: Genomics Institute of the Novartis Research Foundation, 10675 John Jay Hopkins Drive, San Diego, CA 92121.

(1) Gomez, T. M.; Spitzer, N. C. *J. Neurobiol.* **2000**, *44*, 174−83.
(2) Palmer, A. E.; Tsien, R. Y. *Nat. Protoc.* **2006**, *1*, 1057−65.
(3) Tour, O.; Adams, S. R.; Kerr, R. A.; Meijer, R. M.; Sejnowski, T. J.; Tsien, R. W.; Tsien, R. Y. *Nat. Chem. Biol.* **2007**, *3*, 423−31.
(4) Berridge, M. J. *J. Physiol.* **1997**, *499* (Pt 2), 291−306.
(5) Marchant, J. S.; Parker, I. *EMBO J.* **2001**, *20*, 65−76.
(6) Torok, K.; Wilding, M.; Groigno, L.; Patel, R.; Whitaker, M. *Curr. Biol.* **1998**, *8*, 692−9.
(7) Zacharias, D. A.; Baird, G. S.; Tsien, R. Y. *Curr. Opin. Neurobiol.* **2000**, *10*, 416−21.
(8) Adams, S. R.; Tsien, R. Y. *Annu. Rev. Physiol.* **1993**, *55*, 755−84.
(9) Adams, S. R.; Lev-Ram, V.; Tsien, R. Y. *Chem. Biol.* **1997**, *4*, 867−78.
(10) Ellis-Davies, G. C.; Kaplan, J. H. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 187−91.
(11) Barth, A.; Martin, S. R.; Corrie, J. E. *Photochem. Photobiol. Sci.* **2006**, *5*, 107−15.
(12) Kantevari, S.; Hoang, C. J.; Ogrodnik, J.; Egger, M.; Niggli, E.; Ellis-Davies, G. C. *ChemBioChem* **2006**, *7*, 174−80.

10.1021/jo7019898 CCC: $40.75
Published on Web 12/12/2007

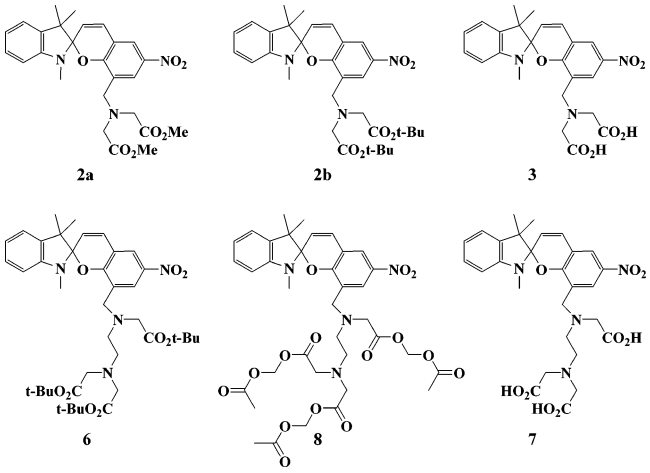
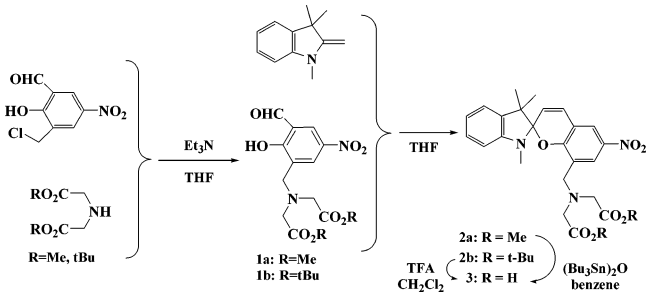


FIGURE 1. Structures of di- and tricarboxyl-substituted photochromes: dicarboxyl-bearing photochromes **2a** (methyl ester), **2b** (*tert*-butyl ester), and **3** (free acid); tricarboxyl-bearing photochromes **6** (*tert*-butyl ester), **7** (free acid), and **8** (acetoxymethyl (AM) ester).

SCHEME 1. Synthesis of NitroBIPS-8-DA (3)



would undergo rapid and reversible, optically driven transitions between two distinct states that exhibit marked differences in their affinity for the metal ion. In addition, one of the two states of the chelate should incorporate a fluorescence readout in order (1) to determine the state of the switch at any time or location in the cell, (2) to measure the free Ca^{2+} level, (3) to quantify the rate and yield of the reaction, and (4) to assess effects of adverse photochemistry such as photobleaching of the chelate.

Nitrobenzospiropyran (nitroBIPS) exhibits many of the idealized features outlined above including a well-documented ability to undergo rapid and reversible, high quantum yield, optically driven transitions between a colorless spiro (SP) state and a colorful merocyanine (MC) state without the release of photoproducts.[13−16] Significantly, the MC state can also decay from its excited state with the emission of red fluorescence. Up to now several potential chelating nitroBIPS probes which harbor polar chelating groups[17] or crown ethers[18−21] have been

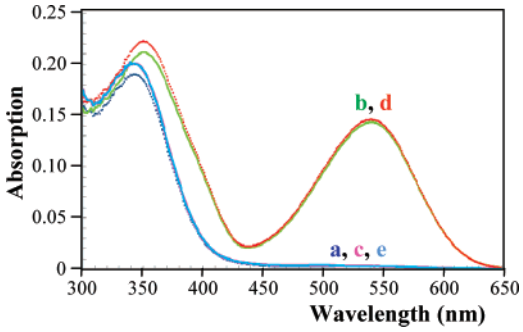
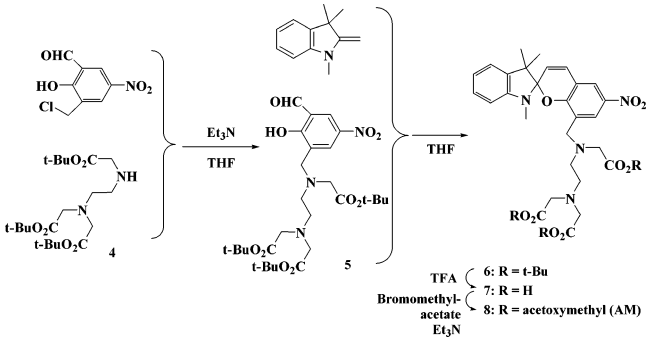


FIGURE 2. Absorption spectra of nitroBIPS-8-*tert*-butyl ester (**2b**) (10 μM) in 1,2-propandiol after alternate irradiation with 365 nm light (curves b and d) for 15 s and 546 nm light (curves a, c, and e) for 30 s.

SCHEME 2. Synthesis of NitroBIPS-8-triA (7) and Its AM Ester (8)



described. For example, Kimura et al.[21] showed that optical transitions between the thermally stable SP and MC states of a crowned spirobenzothiapyran could be controlled in acetonitrile and that the MC state was stabilized by binding lithium ions. All of these studies, however, were limited to investigations in organic solvent and were complicated by thermal, metal ion, and specific solvent effects on the equilibrium between SP and MC states. Chelates for di- and trivalent metal ions that undergo optically driven, rapid, and reversible SP−MC transitions in aqueous solution and within living cells have not been reported. Kimura's group[22] also demonstrated that the open state of chromene crown ethers exhibited an improved affinity for monovalent and specific alkali earth metal ions compared to the closed state, but again these studies were limited to organic solvent.

On the basis of these considerations, we designed and synthesized a new family of optical chelates for reversible control of metal ion binding in vitro and in vivo. Chelating groups were built into the nitroBIPS molecule on the 8-position by using a synthetic approach described in Sakata et al.[13] These chelate-harboring nitroBIPS and their ester derivatives are studied to demonstrate their potential for optical control of metal ions in cells and for in vitro sensing of physiological relevant

(13) Sakata, T.; Yan, Y.; Marriott, G. *J. Org. Chem.* **2005**, *70*, 2009−13.
(14) Sakata, T.; Yan, Y.; Marriott, G. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 4759−64.
(15) Inouye, M. *Mol. Cryst. Liq. Cryst. Sci. Technol.* **1994**, *246*, 169−172.
(16) Kobatake, S.; Irie, M. In *Photochromes. Annu. Rep. Prog. Chem., Sect. C* **2003**, *99*, 277−313.
(17) Deligeorgiev, T.; Minkovska, S.; Jejiazkova, B.; Rakovsky, S. *Dyes Pigm.* **2002**, *53*, 101−108.
(18) Ahmed Saleh, A.; Tanaka, M.; Ando, H.; Iwamoto, H.; Kimura, K. *Eur. J. Org. Chem.* **2003**, 2437−2442.
(19) Salhin Abdussalam, M. A.; Tanaka, M.; Kamada, K.; Ando, H.; Ikeda, T.; Shibutani, Y.; Yajima, S.; Nakamura, M.; Kimura, K. *Eur. J. Org. Chem.* **2002**, 655−662.
(20) Tanaka, M.; Ikeda, T.; Xu, Q.; Ando, H.; Shibutani, Y.; Nakamura, M.; Sakamoto, H.; Yajima, S.; Kimura, K. *J. Org. Chem.* **2002**, *67*, 2223−7.
(21) Tanaka, M.; Kamada, K.; Ando, H.; Kitagaki, T.; Shibutani, Y.; Yajima, S.; Sakamoto, H.; Kimura, K. *Chem. Commun.* **1999**, *1999*, 1453−1454.
(22) Ahmed, S. A.; Tanaka, M.; Ando, H.; Iwamoto, H.; Kimura, K. *Eur. J. Org. Chem.* **2003**, 2437−2442.

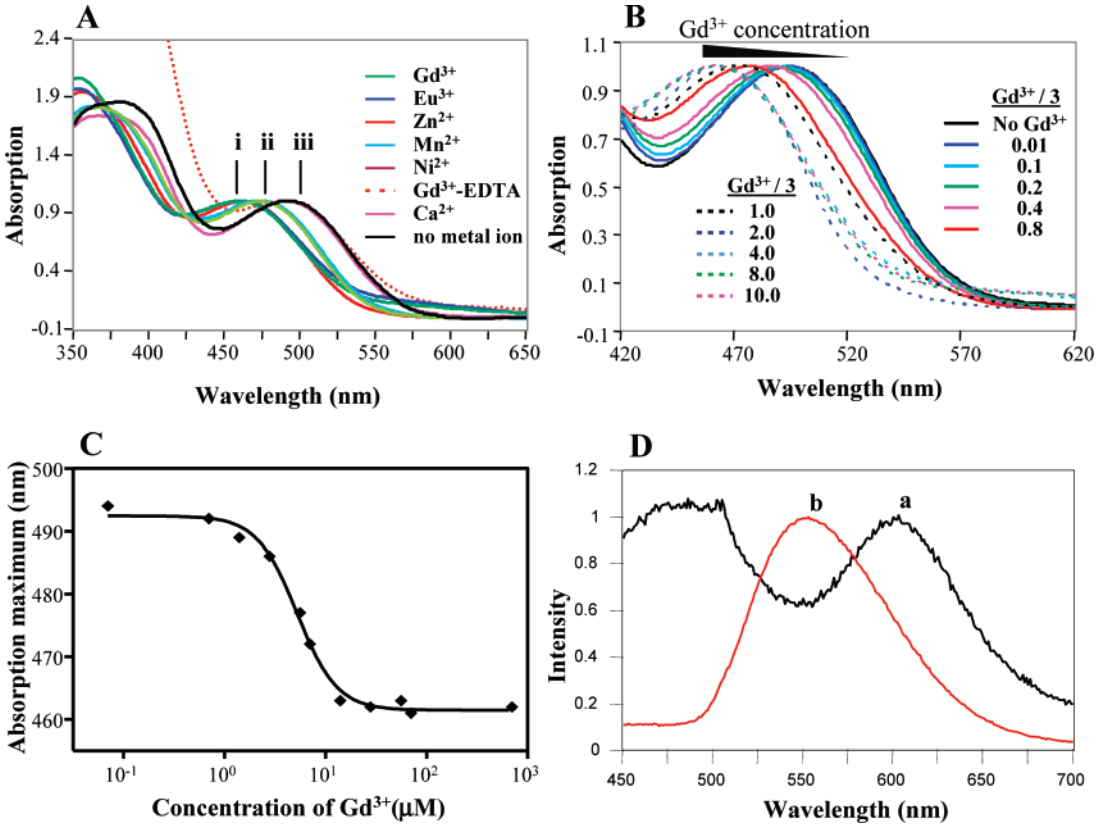


FIGURE 3. (A) Normalized absorption spectra of nitroBIPS-8-DA (**3**) in H_2O at neutral pH in the presence of different metal ions (λ_{max}): (i) Gd^{3+} (459 nm), Eu^{3+} (460 nm), Zn^{2+} (461 nm); (ii) Mn^{2+} (473 nm), Ni^{2+} (473 nm); (iii) Gd^{3+}-EDTA (488 nm), Ca^{2+} (490 nm), no metal ion (492 nm). (B) Titration of **3** with Gd^{3+}. Normalized absorption spectra of **3** (6.9 μM) in H_2O with Gd^{3+} at the ratio of Gd^{3+}/**3**: no Gd^{3+}, 0.01, 0.1, 0.2, 0.4, 0.8, 1, 2, 4, 8, and 10 (right to left). (C) Plot of the change in the absorption maximum wavelength of the $GdCl_3$ complex. The data are fit to a single binding site for Gd^{3+} with a dissociation constant of 5.2 μM. (D) Normalized, fluorescence emission spectra of **3** in H_2O (6.9 μM) in the following conditions: curve a, **3**; curve b, **3** with $GdCl_3$ (6.4 μM). The emission maxima occur at ~600 and ~550 nm for curves a and b, respectively.

metal ions such as Zn^{2+} [23] and Gd^{3+}, the latter being used as a contrast enhancing agent for MRI.[24] Furthermore, the usefulness of MC-fluorescence as a sensitive readout of the efficiency and rate of optical switching in vitro is examined. These studies also led to the new discovery that the SP state of the nitroBIPS is efficiently excited with 2-photon light (700−730 nm) to form the MC state. The nitroBIPS probes described in this report represent our progress toward the design of optical switch chelates that provide rapid and reversible optical control of calcium and other physiologically relevant ions within living cells.

Results and Discussion

Design and Synthesis of Optically Switchable Chelates. The metal ion binding function was built into the nitroBIPS photochrome by introducing a di- or tri-carboxylate-bearing side group at the 8-position (Figure 1 compounds **3** and **7** respectively). First, ester-bearing nitroBIPS (**2a**, **2b**, **6**) were synthesized based on the published methods,[13] as summarized in Schemes 1 and 2 and detailed in the Supporting Information. Combinatorial in design, the syntheses involved a coupling reaction of indoline derivatives with ester-bearing salicylaldehydes. This approach allows for the introduction of carboxyl groups at almost any site on the nitroBIPS scaffold. In this study salicylaldehydes (**1a**, **1b**, **5**) prepared from commercially available 3-chloromethyl-5-nitrosalicylaldehyde were coupled with 1,3,3-trimethyl-2-methyleneindoline to yield the corresponding esters (**2a**, **2b**, **6**).

Next, the ester groups were converted to carboxylic acids. Because conventional saponification was unsuccessful possibly due to the sensitivity of nitroBIPS to strong base, two different hydrolytic reactions were used to generate the free carboxylic acid forms (**3**, **7**): the first is bis(tributyltin)oxide-mediated hydrolysis of the methyl ester (**2a**), and the second is based on trifluoroacetic acid (TFA)-mediated hydrolysis of the *tert*-butyl esters (**2b**, **6**). The obtained chelates **3** and **7** were subjected to Chelex column to remove possible trace amounts of metal cation prior to spectroscopic studies. NitroBIPS-triA (**7**) was further converted to acetoxymethyl ester (AM ester) (**8**) by coupling with bromomethyl actate.

Spectroscopic and Optical Switching Properties of NitroBIPS-8-*tert*-butyl Ester (2b). The absorption spectra of the SP state (SP absorption spectra) of *tert*-butyl ester (**2b**) in 1,2-propandiol has the maximum value at 340 nm and the half-maximal at 375 nm (Figure 2, curve a) and is largely insensitive to solvent polarity whereas the absorption spectrum of the highly polar MC state of **2b** is sensitive to general and specific solvent effects (data not shown), which is consistent with our previous results.[13] Optically driven transitions between the SP and MC states of **2b** occur with high efficiency in organic solvents as

(23) Burdette, S. C.; Walkup, G. K.; Spingler, B.; Tsien, R. Y.; Lippard, S. J. *J. Am. Chem. Soc.* **2001**, *123*, 7831−41.
(24) Cohen, S. M.; Xu, J.; Radkov, E.; Raymond, K. N.; Botta, M.; Barge, A.; Aime, S. *Inorg. Chem.* **2000**, *39*, 5747−56.

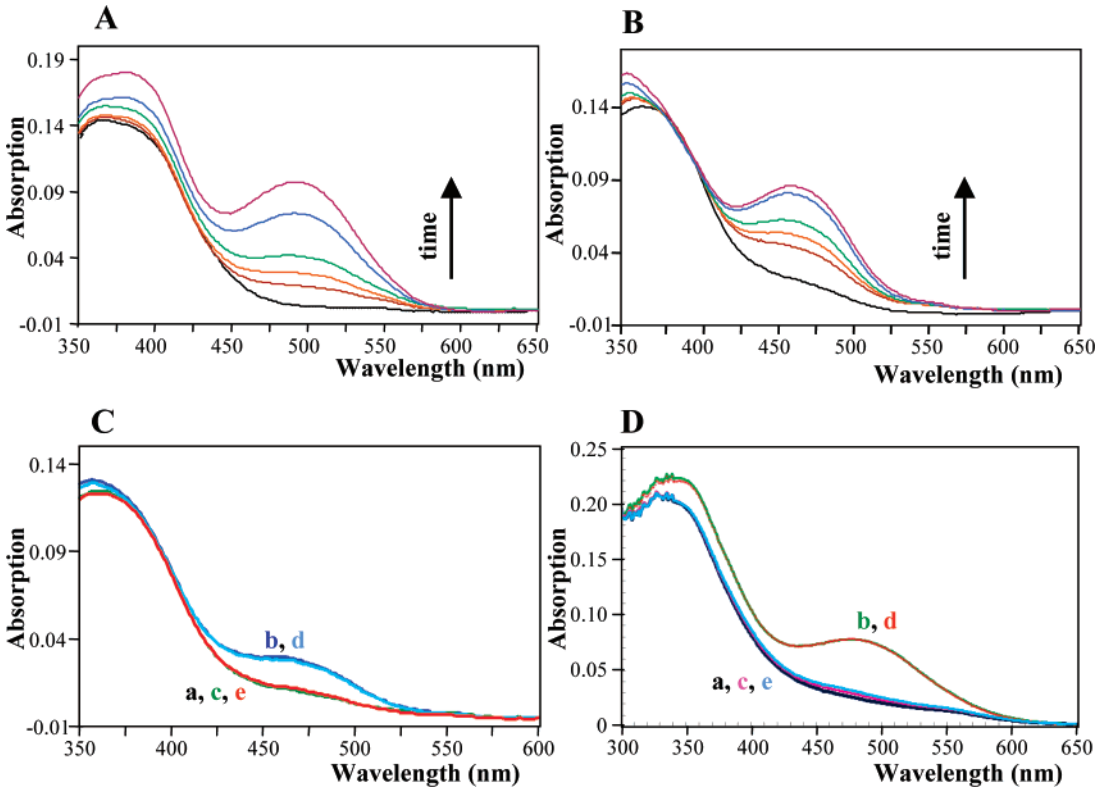


FIGURE 4. (A) Time course of the thermal transition (SP to MC) of nitroBIPS-8-DA (**3**) (4 μM) in the absence of $GdCl_3$ in water (λ_{max} at 491 nm at 14 h). Each spectrum was recorded at the following time points: 15 min; 30 min; 1 h; 3 h; and 14 h. (B) Time course of the thermal transition (SP to MC) of **3** (4 μM) in the presence of 6.4 μM of $GdCl_3$ (λ_{max} at 459 nm at 14 h) in H_2O. Each spectrum was recorded at the following time points: 15 min; 30 min; 1 h; 3 h; and 14 h. (C) Optical switching between the SP and MC states of **3** (4 μM) with the presence of $GdCl_3$ (6.4 μM) in H_2O. Alternate irradiation was performed with 365 nm light (curves b and d) for 15 s and 546 nm light (curves a, c, and e) for 1 min. (D) Optical switching between the SP and MC states of **3** (4 μM) in 1,2-propandiol/Tris, pH 7 (99:1) at 20 °C. Alternate irradiation was performed with 365 nm light (curves b and d) for 15 s and 546 nm light (curves a, c, and e) for 30 s.

shown in Figure 2; the SP to MC transition for a 10 μM solution is complete within a 15 s irradiation of 365 nm light (UV), while the MC state is converted to the SP state following 30 s of irradiation with 546 nm light (VIS). The SP and MC states can be interconverted over many UV−vis irradiation cycles without significant changes in the absorption spectrum or evidence of fatigue (Figure 2).

Metal Ion Chelating Properties of NitroBIPS-8-DA (3) in Water. Conformational change of nitroBIPS and the effect of chelation on its electron density distribution result in the shift on the compound's absorption spectrum. Figure 3A shows that di- and trivalent metal ions bind to the MC state of nitroBIPS-8-DA (**3**) over a μM concentration range. The value of the maximum MC-absorption wavelength was shifted from 492 nm for free acid **3** (i.e., without metal ions) to 459 nm with the presence of 100 μM Gd^{3+} in water, and the range of this spectral shift was specific for each chelated metal ion.

Gadolinium Ion Complexes of NitroBIPS-8-DA (3) in Water. Metyl ester **2a** does not exhibit any significant affinity for Gd^{3+}, which caused the largest shift of nitroBIPS-8-DA (**3**), or divalent metal ions, nor do other nitroBIPS lacking a carboxyl group described by Sakata et al.[13] (data not shown). Also, Gd^{3+} does not show any sensible binding to the SP state of nitroBIPS-8-DA (**3**) in water up to a concentration of 1 mM as might be detected by monitoring changes in the SP-absorption spectrum (data not shown). On the other hand, the MC state of **3** was shown to bind to a single Gd^{3+} ion with a dissociation constant of 5.2 μM in water (Figure 3B,C). Interestingly, the MC state of **3** also exhibits a fluorescence emission whose energy and quantum yield is metal ion specific, as shown for the effect of Gd^{3+} in Figure 3D (curve a, Gd^{3+} free; curve b, with the presence of Gd^{3+}).

NitroBIPS-8-DA (**3**) and related chelates having one or more carboxyl groups in close proximity to the spiro bond exhibit a pronounced reverse photochromism; they undergo a SP to MC transition even in the dark (Figure 4A,B). The origin of reverse photochromism is not fully understood, although nitroBIPS lacking a carboxyl group also show his behavior in acidic medium.[25] Our results suggest that the built-in carboxyl group might accelerate the SP to MC transition by stabilizing protonation of the generated oxy-anion. The rate of reverse photochromism for nitroBIPS-8-DA (**3**) is not significantly affected by the presence of Gd^{3+} over the initial phase of the measurement period (Figure 4A,B).

Absorption studies show that the UV-driven SP to MC transition of nitroBIPS-8-DA (**3**) does occur in water but with low efficiency (Figure 4C). Thus, the SP state of **3** in the presence of chelated Gd^{3+} (Figure 4C, curve a) led to a slight increase in the intensity of the MC-absorption when irradiated for 1 min with 365 nm light (Figure 4C, curve b). By considering the large increase of absorption observed during the thermal transition (Figure 4A,B), this result suggested that the UV-driven SP to MC transition was incomplete. However, the UV-driven

(25) Raymo, F. M.; Giordani, S. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 4941−4944.

MC state can be brought back to the SP state by using 543 nm irradiation, and additional UV-irradiation of the SP state (Figure 4C, curve c) followed by visible light excitation showed that transitions between the SP state (Figure 4C, curves a, c, and e) and the MC-state (Figure 4C, curves b and d) are reversible.

Optical and Thermo-optical Manipulation of NitroBIPS-8-DA in Organic Solvents. In contrast to studies performed in water, nitroBIPS-8-DA (**3**) dissolved in 1,2-propandiol/water (99:1) at pH 7.0 undergoes robust, high fidelity, 365 and 546 nm-driven transitions between the SP and MC states respectively in the presence (Figure 4D, curves a−e) or absence of Gd^{3+} (data not shown). A comparison of the results shown in parts C and D of Figure 4 suggests that water plays a strong role in decreasing the UV-driven quantum yield for the SP to MC transition and promoting reverse photochromism in nitroBIPS-8-DA. The low quantum yield for the UV-driven SP to MC reaction is not unexpected since the reaction is greatly influenced by solvent polarity even for non-carboxyl-bearing nitroBIPS.[26] On the other hand the strong reverse photochromism found for carboxyl-bearing nitroBIPS switches in water suggests that carboxyl groups play either a direct or an indirect role in stabilizing the oxy-anion of the MC state. This complicating factor might be significantly reduced by locating the carboxyl groups away from this region of the nitroBIPS molecule.

Optical Manipulation of Chelates within Living Cells. The AM-ester (acetoxymethyl ester, **8**) of nitroBIPS-8-TriA (**7**) and ethyl esters of other chelate bearing nitroBIPS are capable of diffusing across the plasma membrane of living cells. On the basis of other studies with AM-esters of fluorescent calcium indicators,[8] the AM-ester groups of **8** are presumably hydrolyzed by intracellular esterases to generate the membrane impermeable carboxylate **7**. For example, live NIH 3T3 cells treated with 10 μM of the AM-ester (**8**) in growth medium for 30 min exhibited a strong and uniform MC-fluorescence in cells upon excitation with 535 nm light (Figure 5A). Similar results were obtained for the ethyl ester of a tetracarboxyl-substituted nitroBIPS (data not shown). Support for the claim that AM and ethyl esters of optical switch chelates are hydrolyzed by intracellular esterases was obtained from a separate study using the methyl ester **2a**, which rapidly traverses the plasma membrane but is not a substrate for esterases, and so the intracellular MC-fluorescence of **2a** rapidly disappears from the cell upon washing with fresh medium (data not shown).

Optical switching of nitroBIPS-8-TriA (**7**) within living NIH 3T3 cells was demonstrated by using a 2-photon fluorescence microscope. Irradiation of cells with 543 nm light was used to image the distribution of the MC form of the chelate in cells and to quantify optical switching between the SP and MC states. Thus, quantifying the change in red MC-fluorescence during optical switching allowed us to show that several 543 nm scans of the cell decreased the MC-fluorescence to a baseline value as a result of the MC to SP transition (see montage in Figure 5A). The SP to MC transition was triggered within the same cell by scanning the field with 720 nm light, which led to a 2-photon-mediated excitation of the SP state and photochemistry to the MC state. The laser power of the 720 and 535 nm lasers used in these experiments was low and so several scans were required for complete interconversion of the SP and MC states. On the other hand, other studies using higher 2-photon excitation power showed that a single scan was sufficient to quantitatively convert SP to MC within the focal plane (data not shown).

(26) Görner, H. *Phys. Chem. Chem. Phys.* **2001**, *3*, 416−423.

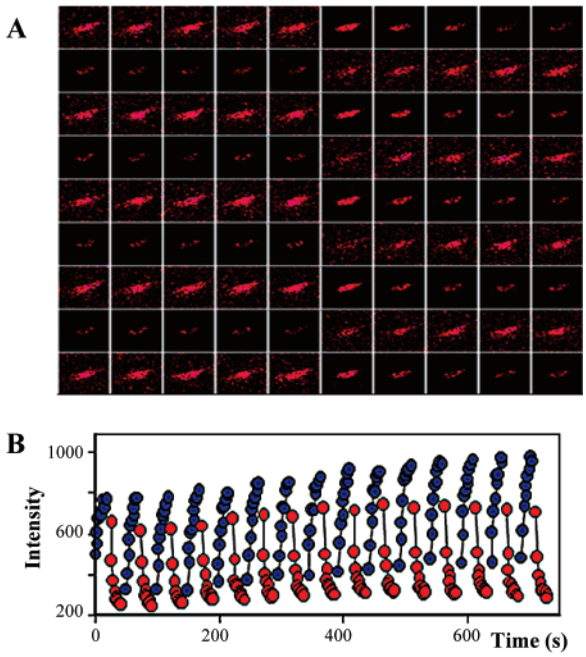


FIGURE 5. (A) A montage of MC-fluorescence images of NIH 3T3 cells loaded with the AM-ester (**8**) of optical switch chelate obtained by confocal imaging using an excitation wavelength of 535 nm and emission >590 nm. The chelate is retained in cells after several washings with culture medium. The MC state was generated by serial scannings of the image field with 720 nm (2-photon) light. (B) The intensity of MC fluorescence in a selected area of the image was used to show the reversibility of transitions between the two states of nitroBIPS-8-TriA (SP to MC, 720 nm, blue circles; MC to SP, 535 nm, red circles). The uniform distribution of the MC fluorescence suggests that the nitroBIPS-8-TriA (**7**) is contained within the cytoplasm. Rapid and reversible, high-fidelity transitions between the SP and MC states are shown for 10 cycles.

Imaging and analysis of the intracellular MC fluorescence of chelate **7** as a function of 2-photon and 543 nm irradiation shows that the chelate undergoes numerous transitions between the SP and MC states in response to alternating irradiation with 720 and 543 nm light (Figure 5A,B). The fastest 720 nm, 2-photon scan possible in our system showed that the SP to MC reaction was complete within the 6 μs pixel dwell time of the laser (data not shown). The gradual increase in MC-fluorescence evidenced prior to 2-photon excitation of each irradiation cycle (Figure 5B) suggests that the power of the 543 nm laser used in this study was too low to convert the entire MC population to SP. The data shown in Figure 5B also show that the optically switchable chelate (**7**) exhibits little fatigue over the 30 cycles of alternate 2-photon/1-photon scanning.

The results shown in this study suggest that nitroBIPS-8-TriA (**7**) unexpectedly undergoes normal photochromism in cells and, unlike nitroBIPS-8-DA in water, it exhibits a robust 2-photon-driven transition between the SP and MC states. It is long known that anionic, aromatic dyes such as fluorescent calcium ion indicators, i.e., Fura-2, bind to cell proteins, and in some cases this leads to a change in the functional properties. Interestingly, the quantum yield for the SP to MC transition of the cell permeable optical switch chelates used in this study is improved in living cells. We speculate that chelate **7** engages in similar nonspecific interactions with cell proteins overcoming reverse photochromism, presumably by shielding the probe from bulk water.

Conclusion

This study introduced new concepts and principles in the design of optically switchable chelates for rapid and reversible 1- and 2-photon mediated control of metal ions in solution and within living cells. By incorporating the iminodiacetate group into the photochromic nitroBIPS scaffold, we have developed chelates that can take two structurally different states, SP and MC. The synthesis involves a coupling reaction of indoline and ester-bearing salicylaldehyde followed by hydrolysis of the ester groups, and this approach can easily be adapted to incorporate a variety of chelating groups on different positions on the BIPS scaffold. The ability to change the relative positions of the chelating carboxyl groups and generated oxyanion between the SP and MC states provides a simple and powerful approach to optically modulate the metal ion binding affinity of the nitroBIPS probe. In vitro studies showed that the MC state of nitroBIPS-8-DA was an effective chelator of Gd^{3+}, and other metal ions that may be distinguished on the basis of the absorption and emission properties of each respective complex. The nitroBIPS harboring ethyl and acetoxymethy esters were shown to cross the cell membrane and were retained following esterase-mediated hydrolysis. Optical switching between the SP and MC states of the chelate and associated modulation of metal ion affinity was demonstrated in vitro and in living cells by using 1- and 2-photon excitation for the SP to MC transition and 543 nm for the MC to SP transition. Given the relatively low affinity of the MC state of the nitroBIPS-8-DA chelatefor Ca^{2+}, we did not expect to see any Ca^{2+} related response of the cell during optical control of the SP and MC states of the related chelate **7**. However, these studies clearly demonstrate the feasibility of controlling and imaging cell Ca^{2+} by using probes related to chelate **7** whose MC state exhibits a higher affinity for Ca^{2+}.

To the best of our knowledge this is the first description of 2-photon excitation-mediated SP to MC transition in nitroBIPS related switches. This property opens up the opportunity to control the two states of nitroBIPS related optical switch by using only 2-photon excitation, i.e., 720 nm for the SP to MC transition and 980 nm for the MC to SP transition. This feature should prove useful in studies that require control of metal ions within tissue slices and under the skin of animals.

Experimental Section

Synthesis. All reagents and starting materials except for triester **4** are commercially available. **4** was synthesized according to Achifelu et al.[27]

General Synthetic Method of Salicylaldehyde (1a, 1b, 5). To a THF solution (8 mL) of di-*tert*-butyl iminoacetate (504 mg, 2.1 mmol) and Et_3N (540 μL, 3.9 mmol) was added 3-(chloromethyl)-5-nitrosalicylaldehyde (435 mg, 2.0 mmol). After refluxing for 4 h, the reaction mixture was filtered and concentrated to give a yellow oil of **1b** as a mixture with Et_3N (**1b**:Et_3N = 10:7), which was used for the subsequent reaction without further purification.

3-[*N*,*N*-Bis(methyloxycarbonylmethyl)aminomethyl]-5-nitrosalicylaldehyde (1a). ^{1}H NMR ($CDCl_3$) δ 3.62 (s, 4H), 3.78 (s, 6H), 4.12 (s, 2H), 8.35 (d, J = 2.7 Hz, 1H), 8.61 (d, J = 2.7 Hz, 1H), 10.36 (s, 1H).

3-[*N*,*N*-Bis(*tert*-butyloxycarbonylmethyl)aminomethyl]-5-nitrosalicylaldehyde (1b). ^{1}H NMR ($CDCl_3$) δ 1.50 (s, 18H), 3.46 (s, 4H), 4.07 (s, 2H), 8.22 (d, J = 3.8 Hz, 1H), 8.65 (d, J = 3.8 Hz, 1H), 10.44 (s, 1H).

General Synthetic Method of NitroBIPS (2a, 2b, 6). A THF solution (2 mL) of salicylaldehyde **1a** (75 mg, 0.22 mmol) and 1,3,3-trimethyl-2-methyleneindoline (51 mg, 0.29 mmol) was stirred at rt for 2 h. Following the addition of saturated Na_2CO_3 the product was extracted with CH_2Cl_2 and dried over Na_2SO_4 and after evaporating solvent, the residue was subjected to column chromatography (SiO_2; eluent, hexane:AcOEt = 5:1) to afford **2a** (51 mg, 35% based on indoline).

NitroBIPS-8-dimethyl Ester (2a). Yield 35% based on indoline; MS (EI) 495 (M^+, 5), 422 (2), 84 (100); HRMS (EI) M^+495.1998 (calcd 495.2006); ^{1}H NMR ($CDCl_3$) δ 1.20 (s, 3H), 1.27 (s, 3H), 2.69 (s, 3H), 3.27 (s, 4H), 3.60 (s, 3H), 3.59 (d, J = 14.2 Hz, 1H), 3.66 (d, J = 14.2 Hz, 1H), 5.87 (d, J = 10.1 Hz, 1H), 6.54 (d, J = 7.6 Hz, 1H), 6.86 (dd, J = 7.6, 7.6 Hz, 1H), 6.92 (d, J = 10.1 Hz, 1H), 7.07 (d, J = 7.6 Hz, 1H), 7.17 (dd, J = 7.6, 7.6 Hz, 1H), 7.94 (d, J = 2.6 Hz, 1H), 8.12 (d, J = 2.6 Hz, 1H).

NitroBIPS-8-di-*tert*-butyl Ester (2b). Yield 63% based on indoline; MS (EI) 579 (M^+, 30), 478 (48), 464 (61), 408 (60), 336 (69), 335 (69), 159 (64), 83 (100); HRMS (EI) M^+579.2948 (calcd 579.2945); ^{1}H NMR ($CDCl_3$) δ 1.19 (s, 3H), 1.27 (s, 3H), 1.38 (s, 18H), 2.70 (s, 3H), 3.23 (s, 4H), 3.61 (s, 2H), 5.85 (d, J = 10.1 Hz, 1H), 6.53 (d, J = 7.4 Hz, 1H), 6.84 (ddd, J = 1.0, 7.4, 7.4 Hz, 1H), 6.92 (d, J = 10.1 Hz, 1H), 7.06 (dd, J = 1.0, 7.4 Hz, 1H), 7.15 (ddd, J = 1.2, 7.4, 7.4 Hz, 1H), 7.92 (d, J = 2.6 Hz, 1H), 8.25 (d, J = 2.6 Hz, 1H).

NitroBIPS-8-tri-*tert*-butyl Ester (6). Yield 30% based on **4**; HRMS (ESI) $[M + H]^+$ 737.7140 (calcd 737.4125); ^{1}H NMR ($CDCl_3$) δ 1.20 (s, 3H), 1.28 (s, 3H), 1.38 (s, 9H), 1.43 (s, 18H), 2.66 (m, 4H), 2.70 (s, 3H), 3.10 (s, 2H), 3.35 (s, 4H), 3.56 (s, 2H), 5.86 (d, J = 10.2 Hz, 1H), 6.54 (d, J = 7.5 Hz, 1H), 6.87(ddd, J = 1.3, 7.5, 7.5 Hz, 1H), 6.93(d, J = 10.2 Hz, 1H), 7.07(dd, J = 0.6, 7.5 Hz, 1H), 7.18 (ddd, J = 1.3, 7.5, 7.5 Hz, 1H), 7.92 (d, J = 3.0 Hz, 1H), 8.22 (d, J = 3.0 Hz, 1H).

NitroBIPS-8-DA (3). From 2a: To a benzene solution (0.5 mL) of **2a** (16 mg, 32 μmol) was added Bu_3SnO (72 mg, 120 μmol), and the reaction mixture was refluxed for 1.5 days. After 10 min of stirring with 0.5 N HCl, the product was extracted with EtOAc, concentrated, and subjected to column chromatography (Sephadex LH-20; eluent, hexane:CH_2Cl_2:MeOH = 2:1:1) to give an impure fraction containing the desired compound. Staring reagents were removed by extraction with CH_2Cl_2, and the aqueous layer was basified and extracted with CH_2Cl_2 to afford **3** (3 mg, 20%). **From 2b:** To a CH_2Cl_2 solution (0.5 mL) of **1b** (40 mg, 69 μmol) was added TFA (0.5 mL), and the reaction mixture was stirred at rt for 3.5 h. After evaporation of the solvent, the residue was subjected to column chromatography (Sephadex LH-20; eluent, hexane: CH_2Cl_2:MeOH = 2:1:1) to afford a red oil **3** (35 mg, 80%). MS (MALDI) 338 (335 + Na), 335 [M − $N(CH_2CO_2H)_2$]; ^{1}H NMR (acetone-d_6) δ 1.25(br s, 3H), 1.36 (br s, 3H), 2.76 (s, 3H), 3.42 (s, 4H), 3.74 (s, 2H), 6.05 (d, J = 10.1 Hz, 1H), 6.63 (d, J = 7.4 Hz, 1H), 6.85 (dd, J = 7.4, 7.4 Hz, 1H), 7.15 (d, J = 7.4 Hz, 1H), 7.16 (dd, J = 7.4, 7.4 Hz, 1H), 7.15 (dd, J = 7.4, 7.4 Hz, 1H), 8.09 (d, J = 2.9 Hz, 1H), 8.27 (d, J = 2.9 Hz, 1H).

NitroBIPS-8-TriAM (8). To a CH_2Cl_2 solution (0.5 mL) of *tert*-butyl ester **6** (10 mg, 15 μmol) was added TFA (0.5 mL), which was then stirred at rt for 5 h. After evaporation of the solvent, the residue was subjected to column chromatography (Sephadex LH-20; eluent, hexane: CH_2Cl_2:MeOH = 2:1:1) to afford a red oil containing nitroBIPS-8-TriA (**7**), which was then dissolved in CH_3CN (1 mL). To this solution were added bromomethylacetate (14 mg, 92 umol) and Et_3N (20 μL, 144 umol) and the mixture was stirred over night. After concentration, the residue was purified on preparative TLC (hexane:EtOAc = 1:1) to afford **8** (2.4 mg, 20% based on **6**). HRMS (ESI) $[M + H]^+$ 785.2890 (calcd 785.2881); ^{1}H NMR ($CDCl_3$) δ 1.20 (s, 3H), 1.28 (s, 3H), 2.12 (s, 6H), 2.18 (s, 3H), 2.64 (s, 4H), 2.70 (s, 3H), 3.20 (s, 2H), 3.50 (s, 4H), 3.57 (m, 2H), 5.67 (s, 2H), 5.73 (s, 4H), 5.88 (d, J = 10.2 Hz, 1H),

(27) Achilefu, S.; Wilhelm, R. R.; Jimenez, H. N.; Schmidt, M. A.; Srinivasan, A. *J. Org. Chem.* **2000**, *65*, 1562−5.

6.54 (d, J = 7.4 Hz, 1H), 6.86 (ddd, J = 1.2, 7.4, 7.4 Hz, 1H), 6.94 (d, J = 10.2 Hz, 1H), 7.07 (dd, J = 0.5, 7.4 Hz, 1H), 7.18 (ddd, J = 1.2, 7.4, 7.4 Hz, 1H), 7.94 (d, J = 2.8 Hz, 1H), 8.09 (d, J = 2.8 Hz, 1H).

Light-Directed Switching. Switching of the probes described in this work was achieved by irradiating the sample (120−1000 μL) with the 365 nm line of a hand-held UV lamp or the 546 nm line of a 100 W Hg-arc lamp.

Microscopy. Single- and two-photon imaging was performed on an LSM510 microscope with a ×40 1.2NA Objective and incorporating a mode-locked titanium:sapphire laser oscillator. Multiphoton photoactivation of the SP state of the optical switch to the MC state was achieved by using 720 nm light. Excitation of the MC state, which leads to its conversion to the SP state or return to the MC ground state with emission of a red photon, was achieved by using 543 nm He−Ne laser excitation. MC-fluorescence was collected through a 565−615 nm band-pass filter with an infrared blocking property.

Cell Studies. Ester derivatives of nitroBIPS-TriA in DMSO were added to the cell medium (DMEM/10% FCS) to achieve a final concentration of ∼10 μM solution. Cells were incubated at 37 °C for 30 min and washed 3−4 times with fresh medium. Loaded cells were scanned sequentially with one switching cycle consisting of 1−5 scans at 720 nm (2-photon) followed by 5−10 scans at 543 nm. The optical switching cycle was repeated 10−30 times for each experiment. Fluorescence of the MC state (and conversion of the MC to SP state) in loaded cells was achieved by scanning a 512 × 512-field with 543 nm light within a second. The SP to MC transition was usually achieved by scanning the field at the same rate with 2-photon light. The optimal excitation wavelength for the SP to MC transition was 715 nm.

Acknowledgment. This work was supported by a grant (5R01EB005217-03) awarded to G.M. We thank Drs. David Piston and Richard Benninger for use of their 2-photon confocal fluorescence microscope.

Supporting Information Available: ^{1}H NMR spectra of the new compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

JO7019898