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Water-Soluble Two-Photon Absorbing Nitrosyl Complex for Light-Activated Therapy through Nitric Oxide Release

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Received August 21, 2007; Revised Manuscript Received December 1, 2007; Accepted December 12, 2007

Abstract: A water-soluble nitrosyl complex with large two-photon absorption was synthesized by incorporating a two-photon absorbing chromophore with tetra(ethylene glycol) units, into the Roussin's red salt. The nitrosyl complex exhibits quenched emission due to energy transfer from the two-photon chromophore to the Roussin's red salt. The nitric oxide (NO) release induced by one- or two-photon irradiation was detected by EPR spectroscopy with a chemical probe, the Fe(II)−*N*-(dithiocarbamoyl)-*N*-methyl-D-glucamine (Fe−MGD) complex. Increased one- or two-photon excited fluorescence, with a concomitant photochemical release of NO, was observed upon one- or two-photon light irradiation. With the observed light-dependent cytotoxicity against cancer cells of the water-soluble nitrosyl complex, it was demonstrated that two-photon-functionalized nitrosyl complexes can be effective NO donors for light-activated treatment.

Keywords: NO release; two-photon absorption; nitrosyl complex; light-dependent cytotoxicity

Introduction

In recent years, increasing attention has been paid to research on nitric oxide (NO) releasing materials (or NO donors), by virtue of the multiple roles playing by NO in biological processes, such as regulation of blood pressure, inhibition of tumor growth, and immunological self-defense.[1,2] As one of the smallest biologically active molecules, cellular NO in nature is almost exclusively generated via a five-electron oxidation of L-arginine, which is catalyzed by NO synthases.[3] However, in the laboratory, researchers have developed and utilized a number of NO-releasing materials such as furoxans, organic nitrite, nitrate, metal nitrosyl complexes, and other nitro compounds.[4–12] Many of the

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Published on Web 02/19/2008

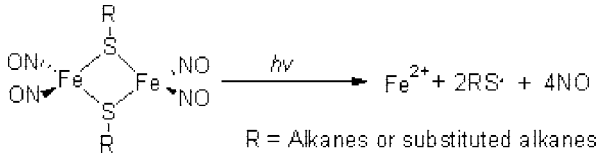

Figure 1. Scheme for photochemical decomposition of a nitrosyl complex.

currently used NO donors such as 1-hydroxy-2-oxo-3-(aminoalkyl)-1-triazenes and 4-alkyl-2-hydroxyimino-5-nitro-3-hexenes are reagents that release NO by spontaneous autolysis.[13,14] Compared to this spontaneous thermally activated NO release, materials with light-controlled NO release stand out as the most promising NO donors, due to the ease of manipulation by light, together with the fast response of the photochemical reactions. Furthermore, light-controlled NO-releasing materials may allow a precise spatial and temporal control of NO delivery. Ford et al.[15–17] have developed some thermally stable metal nitrosyl complexes which can be triggered photochemically to release NO on demand (as shown in Figure 1). As shown in Figure 1, 4 equiv. of NO is released per mole of metal nitrosyl cluster upon photodecomposition. They have successfully incorporated some chromophores (such as fluorescein, AF343) into the backbone of the Roussin's red salt esters. NO was qualitatively demonstrated to be photochemically produced via one-photon excitation, through the use of an NO-specific electrode. They showed that when a chromophore is incorporated into the Roussin's red salt esters, its two-photon absorptivity is retained. For biological applications, it is necessary to make these materials water soluble and biocompatible.

As we know, two-photon techniques in biological applications hold the advantages of a better signal-to-noise ratio due to the reduction of autofluorescence often generated by UV or visible light, and the deeper tissue penetration at the two-photon excitation wavelength in the near IR.[18,19] In recent years, NO donors that provide NO upon exposure to light have drawn special attention because of their utility in photodynamic therapy (PDT) for selected types of cancer.[19] PDT is a noninvasive method of treating malignant tumors and age-related macular degeneration. Current practice of PDT is limited to a few functionalized porphyrins, and some other nonporphyrin photosensitizers, which may generate singlet oxygen ($^1O_2$) upon light irradiation.[20–22] Therefore, there is a significant interest in developing novel and more efficient sensitizers for use in PDT. NO releasing materials may be another class of promising sensitizers for PDT application, besides those materials generating singlet oxygen.

A series of oligo-phenylene vinylenes (OPVs) with comparatively large two-photon absorption (TPA) cross-sections have been prepared.[23,24] The TPA cross-section values for these OPVs, increase with an increase in the π-conjugation length and the degree of intramolecular charge-transfer upon excitation. However, these compounds are barely soluble in water, and they are not suitable for biological applications. Therefore, there is a need to introduce water-soluble functional groups (such as oligo(ethylene glycol)) into the OPV backbone, resulting in materials with large TPA together with good water solubility. As shown in Scheme 1, three groups of tetra(oligo(ethylene glycols) monomethyl ether) make compound **2P-I** highly water

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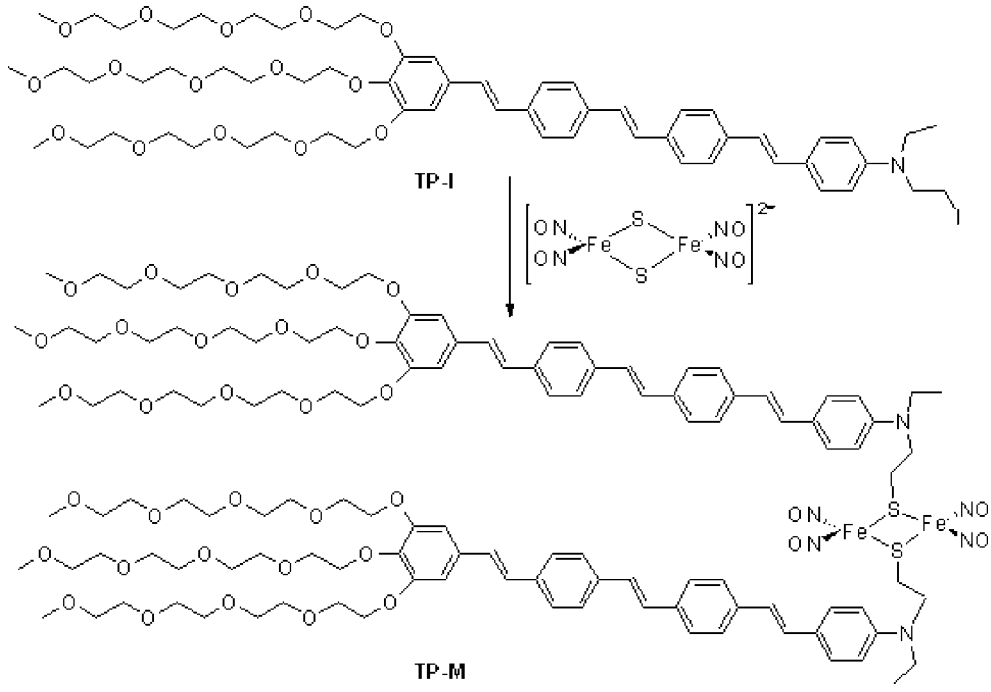
Scheme 1. Synthesis of the Water-Soluble Metal Nitrosyl Complex



soluble, while the iodoalkyl group at the other end can be used to link to a Roussin's red salt ester, resulting in the final targeted NO releasing Roussin's red salt complex (**2P-M**).

We reported here the preparation of this water soluble metal nitrosyl complex with large TPA. We also report on its photochemical and photophysical properties as well as its cellular cytotoxicity under one-, and two-photon excitation.

Results and Discussion

Synthesis. As shown in Scheme 1, the water-soluble metal nitrosyl complex was obtained by reaction between a two-photon absorbing chromophore (**2P-I**) and Roussin's red salt,[25] with 36% yield. The synthetic route for **2P-I** is shown in Scheme 2. It starts with the alkylation reaction between the commercially available tetra(ethylene glycol) monomethyl ether tosylate and 3,4,5-trihydroxybenzaldehyde in the presence of K_2CO_3. Then the Horner−Wadsworth−Emmons reaction between compound **2** and diethyl *p*-vinylbenzylphosphonate[26] gave vinylated compound **4**. Finally, the palladium-mediated Heck coupling reaction between compound **4** and the bromomide (**5**) gave compound **2P-I**. Compounds **2P-I** and **2P-M** are water soluble (up to 5 g/L in water).

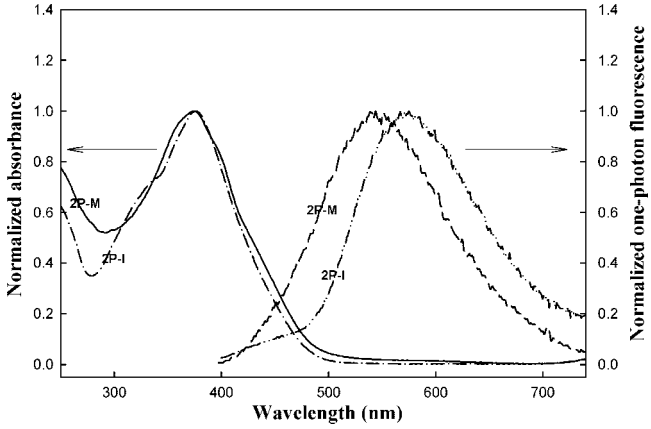


Figure 2. Linear absorption and emission spectra for compounds **2P-I** and **2P-M**.

Linear Absorption and Emission. As shown in Figure 2, compounds **2P-I** and **2P-M** have similar linear absorption spectra. However, compound **2P-M** showed a hypsochromically shifted emission peak at 544 nm, compared to compound **2P-I** (λ_{em} = 575 nm). Table 1 summarizes their photophysical properties, including linear absorption maxima, molar extinction coefficients, emission maxima, and fluorescence quantum yields. As listed in Table 1, compound **2P-I** has a relatively higher fluorescence quantum yield of 0.13, while compound **2P-M** has a much lower fluorescence quantum yield of 0.007. We attribute this reduced emission observed for compound **2P-M** to be due to energy transfer from the two-photon chromophore to Roussin's red salt (Fe−NO unit).

Two-Photon Absorption and Emission. Shown in Figure 3 is the fluorescence spectrum for compound **2P-I** excited at 775 nm. The energy of one photon with 775 nm wavelength is not high enough to excite a **2P-I** molecule from its ground state to the excited state; therefore, more

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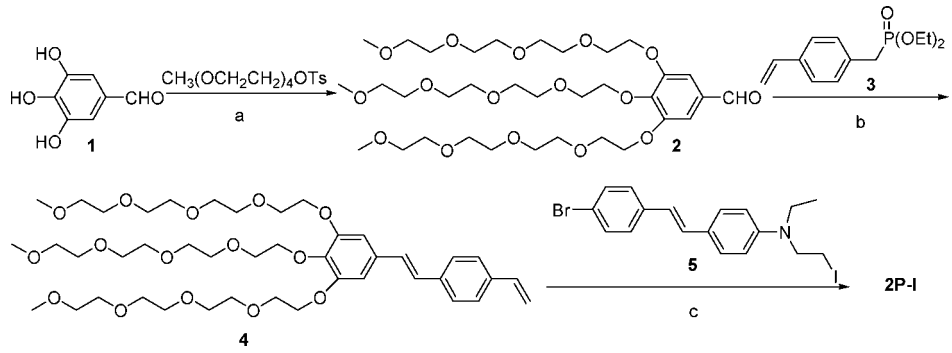
Scheme 2[a]



[a] Reaction conditions: (a) K_2CO_3, DMF, 80 °C, 24 h under Ar, 85%; (b) *t*-BuONa, THF, 0–20 °C, 12 h, 80%; (c) $Pd(OAc)_2$, Et_3N, P(*o*-tolyl)$_3$, CH_3CN, 76 °C.

Table 1. Photophysical Properties of Compounds **2P-I** and **2P-M**

compd	λ_{abs}/nm	ϵ/M^{-1} cm^{-1}	λ_{em}/nm	QY[a]	2PA at 775 nm/GM[b,c]
2P-I	376	4.4×10^4	575	0.13	370
2P-M	375	7.9×10^4	544	0.007	682

[a] Fluorescence quantum yield. [b] GM: Goeppert−Mayer units (1 GM = 10^{-50} cm^4 s $photon^{-1}$). [c] Error limit ±15%.

than one photon is needed for the excitation process. To further prove the nature of this process, the relationship between the fluorescence intensity and the laser intensity (775 nm, fs laser) was investigated. The results are shown in the inset of Figure 3 where a log–log plot shows a slope of 1.92, demonstrating TPA. The two-photon excited fluorescence spectrum (in Figure 3) is basically the same as its one-photon excited fluorescence spectrum (in Figure 2), which confirms that both emissions are from the same excited state.

To further optimize the excitation wavelength for two-photon excitation, the TPA spectrum for compound **2P-I** was

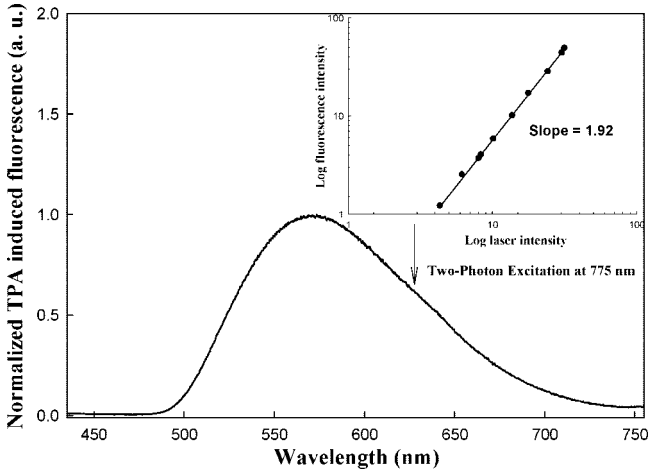


Figure 3. Two-photon excited fluorescence for compound **2P-I** in water; (inset) fluorescence intensity versus the excitation laser intensity.

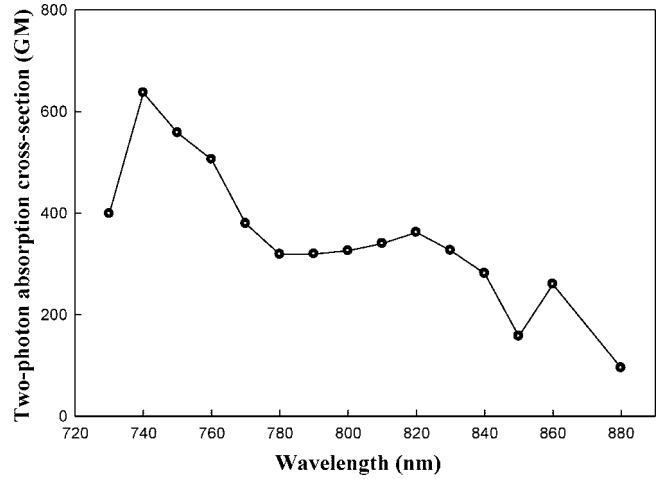


Figure 4. Two-photon absorption spectrum for compound **2P-I** in water.

measured by using the two-photon excited fluorescence method, with Rhodamine 6G as a standard.[27] The results are shown in Figure 4, where one can see that compound **2P-I** has a TPA peak value of 660 GM at 740 nm. In addition to the measurement by the fluorescence method, the TPA cross section of **2P-I** at 775 nm was also determined by an independent absorption method (δ_{775} nm = 370 ± 55 GM) using a standard (AF350) with known δ.[28] Both values for δ are in good agreement, considering the different nature of the methods employed for measurements. The fluorescence quantum yield for compound **2P-M** is quite low, which induces a large uncertainty in the measurement of TPA spectrum using the two-photon excited fluorescence method. Therefore, we measured its TPA properties using the nonlinear transmission method.[28] As shown in Table 1, compound **2P-M** has a moderately large TPA cross section value of 682 GM at 775 nm, close to the value for compound

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2P-I (370 GM), considering compound **2P-M** has two TPA active structural units. These results reveal that when the two-photon absorbing chromophore attaches to Roussin's red salt ester its two-photon absortivity is basically retained.

EPR Analysis. Detection of the short-lived NO released from the compound **2P-M** solution after light irradiation was carried out by an EPR spin-trapping method with *N*-methyl-D-glucamine dithiocarbamate (MGD)−Fe^{2+} complex which reacts with NO to give a stable paramagnetic complex [$(MGD)29_2$−Fe^{2+}−NO].[29] In EPR spectroscopy, the complex is observed as a broadened three-line spectrum. When the compound **2P-M** solution was left in the dark, and subjected to EPR spectroscopy, no EPR signal was detected (Figure 5a). In contrast, when compound **2P-M** solution was photoirradiated by 365 nm UV light (one-photon excitation) for 10 min or by 775 nm laser light (two-photon excitation) for 20 min, and then subjected to EPR spectroscopy, a characteristic three-line EPR signal was observed on the spectrum (Figure 5b,c). As shown in Figure 5b,c, the intensity of EPR signals induced by two-photon irradiation is comparable to that induced by one-photon irrdiation. The EPR spectroscopy results show that compound **2P-M** will release NO upon photoirradiation. Further evidence for net photoreaction was obtained via positive-ion electrospray ionization mass spectroscopy. ESI+ MS spectra of the solutions were obtained before and after 775 nm irradiation with 165 fs pulses. After two-photon excitation, a large peak at *m/z* 1220 was observed with the expected isotopic pattern for a doubly charged iron species. This is consistent with the doubly charged ion $[2P - M - NO]^{2+}$.

Photochemical Properties. When the two-photon absorbing chromophore **2P-I**(antenna) is linked to Roussin's red salt ester (Fe-NO unit), there is an energy transfer from the antenna to the Fe−NO unit, which is responsible for the quenched emission found for compound **2P-M**. However, after light activation, the linkage between the antenna and the Fe−NO unit is disconnected (as shown in Figure 1). Therefore, the pathway for the previous energy transfer disappears, and the resulting antenna chromophore exhibits a higher fluorescence quantum yield as usual. In other words, this NO-releasing process is concomitant with increased fluorescence emission. We measured the one-photon excited fluorescence for compound **2P-M** (0.4 mM in H_2O) versus irradiation time of UV light, and the results are shown in Figure 6. As shown in Figure 6, gradually increased emission was observed with increasing time of UV light irradiation. After 140 min of light irradiation, the fluorescence intensity was enhanced by a factor of 36. Light irradiation for an additional 10 min does not lead to a further increased in emission intensity. At this point, when the solution was subjected to an EPR analysis, no signal was found, demonstrating the completion of the NO release process. The inset

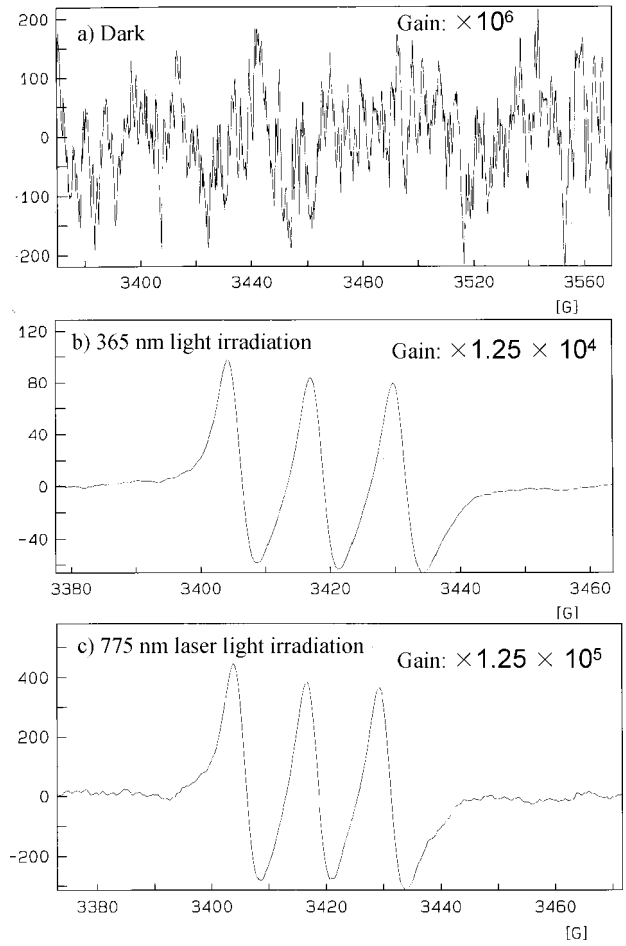

Figure 5. EPR spectra of [$(MGD)_2$−Fe] complex before and after photoirradiation in the presence of compound **2P-M**: (a) in the dark; (b) after photoirradiation with 365 nm UV light for 10 min; (c) after photoirradiation with 775 nm IR laser light for 20 min. Samples contained 0.4 mM compound **2P-M** and 10 mM Fe-MGD in distilled water. EPR spectra were recorded with a modulation amplitude of 2.02 G and a microwave power of 15.9 mW.

in Figure 6 shows that after 30 min of light irradiation (365 nm), the cuvette on the left (a) is highly fluorescent, while the one kept in the dark (b, on the right) is nonfluorescent.

Since compound **2P-M** has a strong TPA at 775 nm, an enhanced emission may also be observed with the concomitant photochemical release of NO, when it is irradiated by 775 nm laser light. Shown in Figure 7 is the one-photon excited fluorescence spectra vs irradiation time of 775 nm laser light. The fluorescence spectra were measured in H_2O with a concentration of 0.4 mM and with an excitation wavelength of 388 nm. Similar to one-photon irradiation, the intensity of the one-photon excited fluorescence spectrum goes up with the increasing time of 775 nm laser irradiation. After 2 h of light irradiation, a 47-fold increase in emission was observed. The increased emission is attributed to the fluorescent species generated by photodissociation with the IR laser.

Similarly, an enhanced two-photon excited fluorescence should be also observed when compound **2P-M** is subjected

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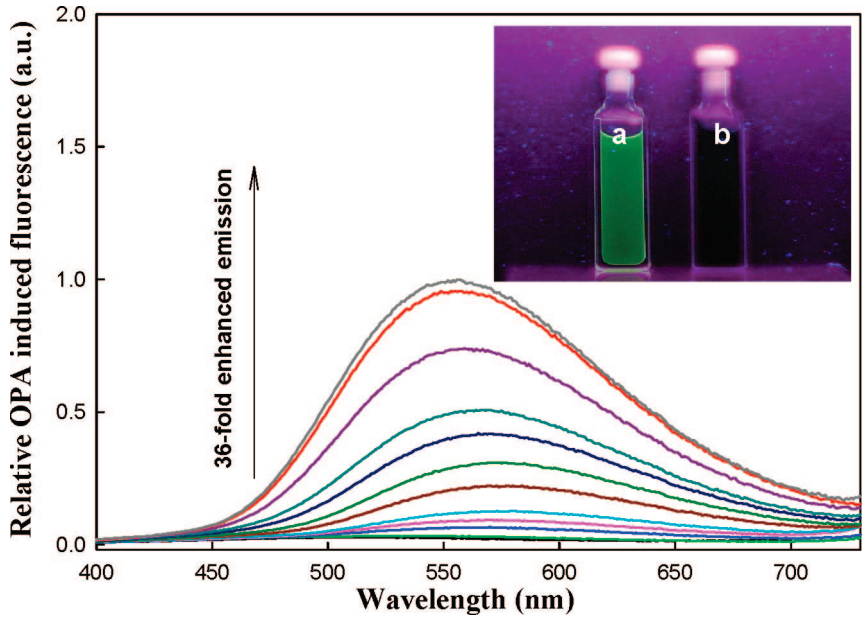

Figure 6. One-photon excited fluorescence spectra after different times of UV light irradiation (irradiation time increased from the bottom to the top: 0, 3, 10, 15, 20, 30, 45, 60, 80, 100, 120, 140 min); (inset) photograph of two aqueous solutions of compound **2P-M** in a cuvette under UV light with (a) and without (b) light irradiation (365 nm) for 30 min.

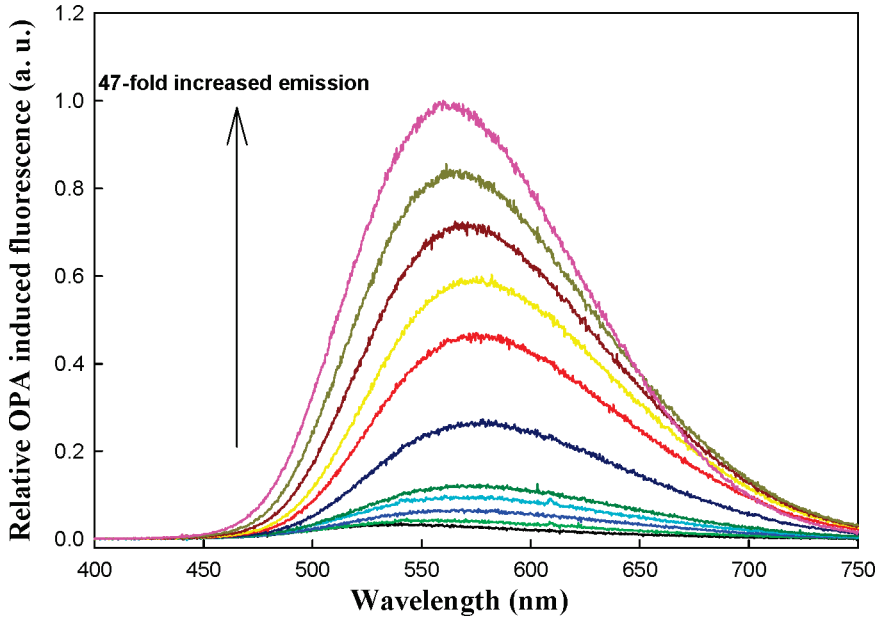

Figure 7. One-photon excited fluorescence spectra after different irradiation times of 775 nm laser (irradiation time increased from the bottom to the top: 0, 5, 10, 20, 30, 50, 70, 80, 90, 100, 120 min).

to IR light irradiation. Figure 8 shows the two-photon excited (775 nm) fluorescence spectra for compound **2P-M** before and after IR laser irradiation (775 nm). A 43-fold increased emission was observed after 110 min of IR light irradiation. Furthermore, we measure the peak intensity of two-photon excited fluorescence versus 775 nm laser irradiation time. The results are shown in the inset of Figure 8, where one can see a monotonic growth of two-photon excited fluorescence in the beginning 110 min. However, after 110 min of light irradiation, the value of fluorescence intensity at 570 nm goes down due to possible photodecomposition induced by intense laser.

Cytotoxicity against Cancer Cells. It has been reported that NO is a mediator of the cytotoxic action of macrophages toward tumor cells, through inhibition of mitochondrial enzyme activity and DNA synthesis.[2c,11] Therefore, we determined whether NO induced from **2P-M** can be used as a cytotoxic agent for some cancer cells.

As shown in Figure 9, only 24% of cells are alive when the Hela cells were treated with compound **2P-M** and then

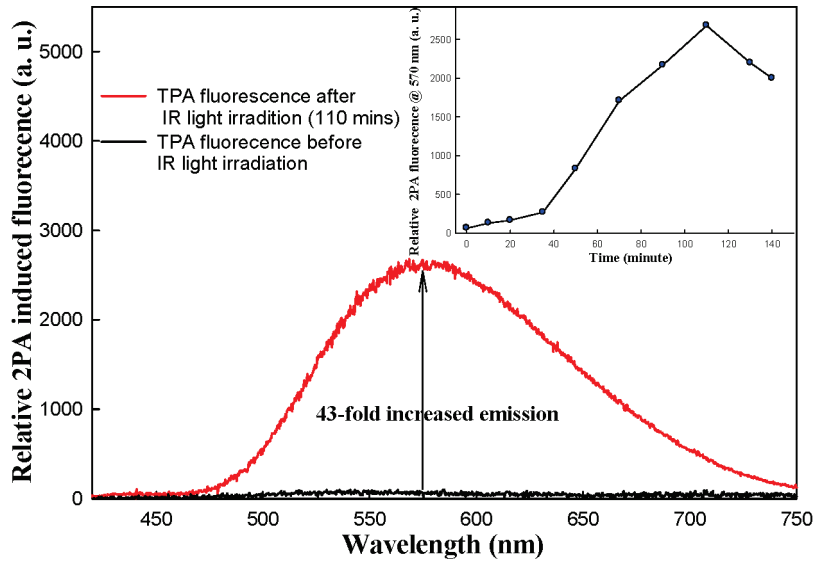

Figure 8. Fluorescence spectra of compound **2P-M** (0.4 mM in H_2O) before and after 110-min IR laser irradiation; (inset) fluorescence intensity at 570 nm versus irradiation time of 775 nm laser light.

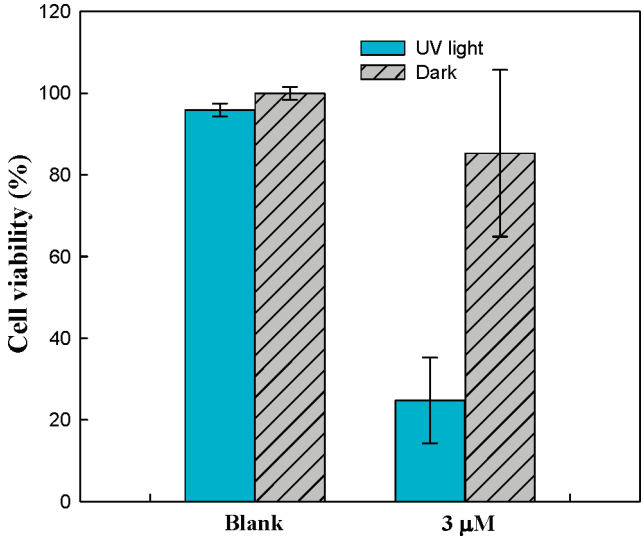

Figure 9. Percentage of cell survival of Hela cells, after treatment with compound **2P-M** and subsequent irradiation with 365 nm UV light for 40 min (with reference to untreated cells under dark as having 100% survival). Cell viability was assayed by the MTT method (values: mean ± standard deviation).

subjected to 40 min of UV light irradiation (365 nm, ~5 mW/cm^2). In contrast, the Hela cells without treatment by compound **2P-M** have 95% survival. Please note that this UV light is harmful to the cells; 5% of cell death is due to exposure to UV light. As shown in Figure 9, a culture dish without **2P-M** treatment and UV light irradiation was used as a control. The results showed that the light-induced NO release can be translated into light-induced cytotoxicity *in vitro*. A control experiment was carried out where the **2P-M** sample was subjected to a 2-h light irradiation (365 nm) before treating the cells. With a similar dose, nearly no cytotoxicity (<2% cell death) was observed, which confirms that the observed cytotoxicity is caused by NO, not by other light induced **2P-M** derivatives. We should point out that the purpose of this experiment is only to establish the feasibility of using light-triggered NO donors for cancer therapy. In practice, UV or visible light cannot penetrate deep into mammalian tissue and would cause more tissue damage. As a result, there is a considerable interest in treatments using lights in the near-IR or IR range.[18]

As we know, the two-photon technique has some advantages over one-photon excitation, such as deeper light penetration in mammalian tissue and less tissue damage by IR irradiation. The present study as well as a previous EPR report has shown that NO release can be triggered not only by one-photon irradiation but also by two-photon irradiation.[17] For the application of the NO donors in a biological study, compound **2P-M** has the advantage of good water solubility and large TPA. Since TPA is a relatively weak process compared to one-photon absorption, it is necessary to increase the concentration of compound **2P-M** up-taken by the cells. In the presence of compound **2P-M** in concentration range from 0 to 40 μM, laser light of 775 nm wavelength (~0.8 W/cm^2) was shed for 5 min on a fixed area of a culture plate containing Hela or Cos-7 cells. The cells were observed after a 24-h incubation at 37 °C under dark. The plate containing no compound **2P-M** was used as a control. As shown in Figure 10, with increasing amount of drug loading, increased cell deaths were found both for Hela and Cos-7 cells. The plates in the dark have higher cells survivals, which indicates that the cytotoxicity was quite dependent on photoirradiation. However, increased concentration of compound **2P-M** also increased its dark cytotoxicity. For example, when the concentration of compound **2P-M** increases from 8 μM, to 16 μM, 24 μM, 32 μM, and to 40 μM, the cell survival of Hela cells after IR irradiation decreases from 53%, to 50%, 46%, 42%, and 35%, respectively. Considering the dark cytotoxicity induced by compound **2P-M**, one may still see IR light induced cell death

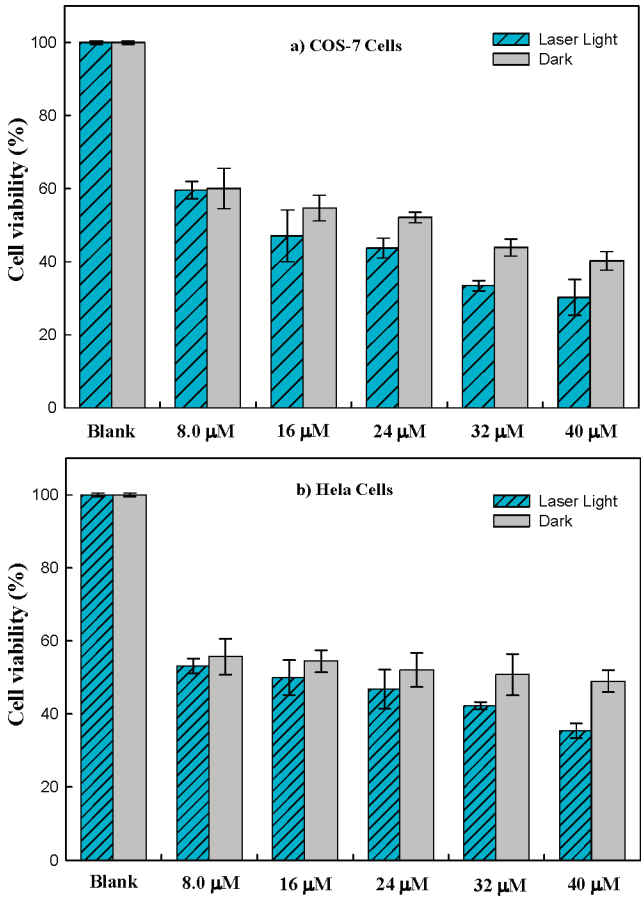

Figure 10. Percentage of cell survival of Cos-7 and Hela cells, after treatment with **2P-M** and subsequent irradiation with 775 nm laser light for 5 min (with reference to untreated cells under dark as having 100% survival). Cell viability was assayed by the Cell Titer-Glo method (values: mean ± standard deviation).

of Hela cells increase from 2.5% to 13.6% when the concentration of compound **2P-M** increases from 8 to 40 μM. Similar effects were also observed for Cos-7 cells. When the concentration of compound **2P-M** loaded in Cos-7 cells increases from 8 to 40 μM, IR light induced cell death increases from 0.4% to 10%. These preliminary results indicate the feasibility of employing two-photon active NO donors for photodynamic therapy application. However, a comparison of Figures 9 and 10 shows that the TPA-induced toxicity is less pronounced than that induced by one-photon absorption. This difference is due to the fact that two-photon excitation is a weak nonlinear process compared to one-photon excitation. To achieve IR light-activated therapy for tumors more effectively, one still needs to design and prepare NO-releasing materials, which have a stronger two-photon response, less dark toxicity, and the ability to pass through tumor cell membranes easily and selectively.

Conclusions

We have described the synthesis and photophysical characteristics of a novel NO donor, where NO release can be controlled by UV or IR light, thus having a significant potential for therapeutic application. The water-soluble metal nitrosyl complex has a quite large TPA cross-section value compared to some commercially available chromophores. The two-photon chromophore basically retained its one-photon and two-photon absorption properties when attached to the metal nitrosyl complex, although it shows a blue-shifted and quenched emission. The NO release induced by one-photon or two-photon irradiation was detected by EPR spectroscopy. The fluorescence quenching and NO labilization can be attributed to the energy transfer from the two-photon chromophore to the Fe−NO unit. After light activation, this compound decomposes to the species with a much higher fluorescence quantum yield. Therefore, an increased emission was observed with increasing time of UV (one-photon) or IR (two-photon) light irradiation. The NO release is concomitant with increased emission; as a result, the NO-releasing process can also be detected by measuring the fluorescence intensity of compound **2P-M**. As the NO produced from compound **2P-M** by photoirradiation can function as a cytotoxic agent, the one-photon induced toxicity to Hela cells was investigated, as well as the two-photon induced cytotoxicity of compound **2P-M** to Hela and Cos-7 cells. The *in vitro* cytotoxicity experiments showed that, in principle, this NO-releasing complex can be used as a cytotoxic agent for cancer therapy in the future. Using near-IR light as the excitation source has advantages of operating deep into mammalian tissue with more precise focusing, since TPA is proportional to the square of the incident radiation intensity.

Experimental Section

Reagents were purchased from Aldrich, Inc., and used without further purification, unless otherwise stated. Compounds **2**, **3**, and **5** and Roussin's red salt were prepared according to literature procedures.[25,26,30,31] ^{1}H NMR spectra were recorded at either 300 or 500 MHz. ^{13}C NMR spectra were recorded at 75 MHz. MALDI-TOF MS spectra were recorded on a Bruker Biflex IV MS spectrometer with dithranol as a matrix. Electrospray ionization (ESI) mass spectra were obtained on a Thermo Finnigan LCQ Advantage mass spectrometer. Linear absorption spectra were recorded on a Shimadzu UV-3101 PC spectrophotometer. One-photon excited fluorescence was measured by using a Jobin-Yvon Fluorolog FL-311 spectrofluorometer. Fluorescence quantum yields for compounds **2P-I** and **2P-M** were determined in water at a concentration of 1×10^{-6} M, with an external

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reference of Coumarin 152 ($\varphi_f = 0.21$ in ethanol).[32] The EPR spectra were measured by a Bruker ESP-300E EPR spectrometer.

Synthesis of Compound 2. 3,4,5-Trihydroxylbenzaldehyde (1.54 g, 10 mmol), tetra(ethylene glycol) monomethyl ether tosylate (15.0 g, 41.4 mmol), and powder K_2CO_3 (11.0 g, 80 mmol) were mixed in 50 mL of DMF. The mixture was heated at 80 °C under argon atmosphere for 24 h. After cooling, the mixture was poured into water and extracted by dichloromethane. The solvent in organic layer was removed under vacuum, and the residue was purified by column chromatography (silica gel, eluents: petroleum ether (PE) then acetyl acetate (EtOAc), and finally with EtOAc/methanol = 1:1). Pure product (5.9 g) as a colorless liquid was collected (79%). ^{1}H NMR (300 MHz, $CDCl_3$, δ): 9.82 (s, 1H), 7.14 (s, 2H), 4.27–4.21 (m, 6H), 3.88 (t, 4H, J = 4.5 Hz), 3.80 (t, 2H, J = 4.5 Hz), 3.75–3.71 (m, 6H), 3.70–3.61 (m, 24H), 3.56–3.52 (m, 6H), 3.37 (m, 9H). ^{13}C NMR (75 MHz, $CDCl_3$, δ): 190.92, 152.96, 131.54, 108.93, 72.47, 71.86, 70.75, 70.52, 70.42, 69.54, 68.90, 58.92. HRMS/ESI: calcd for $C_{34}H_{60}O_{16}Na$ ($M + Na^+$) 747.3774, found 747.3782.

Synthesis of Compound 4. To a solution of **3** (2.54 g, 10 mmol) in anhydrous THF (50 mL) was added sodium *tert*-butoxide (2.24 g, 20 mmol) under argon at 0 °C. To this suspension was added slowly aldehyde **2** (3.74 g, 5 mmol) in anhydrous THF. The mixture was then allowed to warm to room temperature and stirred for 12 h. The mixture was quenched by adding 100 mL of water. Solvents were removed under reduced pressure. The resulting oil was dissolved in dichloromethane, washed with brine (20 mL) and water (20 mL), and dried over magnesium sulfate. The product was purified by column chromatography (CH_2Cl_2/MeOH = 97:3 to 80:20 in volume) to afford **4** (75%). ^{1}H NMR (500 MHz, $CDCl_3$, δ): 7.45 (d, J = 8.5 Hz, 2H), 7.40 (d, J = 8.5 Hz, 2H), 6.97 (d, J = 18.0 Hz, 2H), 6.76 (s, 2H), 6.71 (dd, J_1 = 17.5 Hz, J_2 = 11.0 Hz, 1H), 5.76 (d, J = 17.5 Hz, 1H), 5.25 (d, J = 11.0 Hz, 1H), 4.21(t, J = 5.0 Hz, 4H), 4.16 (t, J = 5.0 Hz, 2H), 3.87 (t, J = 5.0 Hz, 4H), 3.79 (t, J = 5.0 Hz, 2H), 3.75–3.71 (m, 6H), 3.70–3.62 (m, 24H), 3.55–3.52 (m, 6H), 3.38–3.36 (m, 9H). ^{13}C NMR (75 MHz, $CDCl_3$, δ): 152.60, 136.66, 136.59, 136.26, 132.71, 128.20, 127.55, 126.38, 113.52, 106.21, 72.32, 71.82, 70.71, 70.54, 70.48, 70.38, 69.69, 68.84, 58.87. HRMS/ESI: calcd for $C_{43}H_{68}NaO_{15}$ ($M + Na^+$) 847.4450, found 847.4457.

Synthesis of Compound 2P-I. Compound **4** (1.69 g, 2 mmol), compound **5** (1.0 g, 2.2 mmol), $Pd(OAc)_2$ (13 mg, 0.04 mmol), P(*o*-tolyl)$_3$ (190 mg, 0.06 mmol), Et_3N (2.0 mL), and CH_3CN (20 mL) were added to a pressure tube with a plunger valve and a magnetic bar under argon atmosphere. The resulting mixture was refluxed for 24 h and then cooled to room temperature. The mixture was poured into 50 mL of water and extracted several times with dichloromethane. The organic phase was dried by $MgSO_4$ and filtered. The solvent was removed by rotary evaporation. The final product was collected in 84% by column chromatography on silica gel, using CH_2Cl_2/CH_3OH as the eluent. ^{1}H NMR (400 MHz, $CDCl_3$, δ): 7.50–7.40 (m, 10H), 7.08–6.77 (m, 8H), 6.77 (s, 2H), 6.68 (d, J = 8.4 Hz, 2H), 4.23–4.15 (m, 6H), 3.89–3.74 (m, 6H), 3.73–3.37 (m, 51H), 1.22 (t, J = 6.8 Hz, 3H). ^{13}C NMR (75 MHz, $CDCl_3$, δ): 152.38, 146.13, 137.50, 136.87, 136.23, 135.72, 128.47, 128.36, 127.98, 127.91, 127.48, 126.76, 126.29, 125.92, 124.10, 111.71, 105.68, 72.33, 71.74, 71.65, 70.53, 70.41, 70.33, 70.25, 70.12, 69.47, 68.47, 62.13, 58.97, 53.44, 45.07, 16.32, 12.78. HRMS/ESI: calcd for $C_{61}H_{86}INNaO_{15}$ ($M + Na^+$) 1222.4934, found 1222.4921 (100%).

Synthesis of Compound 2P-M. To a 50 mL three-neck flask covered in aluminum foil, equipped with a stir bar, was added a solution of 0.6 g of **2P-I** (5 mmol) in 40 mL of dry, distilled THF. This solution was purged with argon and stirred until the compound was fully dissolved. To this solution was added 0.120 g of $Na_2[Fe_2S_2(NO)_4]\cdot 8H_2O$ (2.5 mmol) under argon. After the round-bottom flask was purged with argon, the mixture was allowed to stir in the dark for 2 days. The reaction was stopped, and the solvent was removed via rotary evaporation. The compound was then extracted several times with 100 mL of a CH_2Cl_2 mixture. The dark organic solutions were combined and then reduced to a minimum volume on the rotary evaporator. The residue was purified by column chromatography (silica gel CH_2Cl_2/CH_3OH) yielding the titled compound (36%). ^{1}H NMR (500 MHz, $CDCl_3$, δ): 7.50–7.40 (m, 20H), 7.11–6.88 (m, 16H), 6.68 (s, 4H), 6.67 (d, J = 8.5 Hz, 4H), 4.23–4.19 (m, 12H), 3.89–3.34 (m, 102H), 1.21 (t, J = 7.0 Hz, 6H). ^{13}C NMR (75 MHz, $CDCl_3$, δ): 152.52, 146.53, 137.51, 136.81, 136.27, 135.70, 128.46, 128.31, 128.02, 127.90, 127.46, 126.73, 126.27, 125.82, 124.05, 111.76, 105.93, 72.31, 71.77, 70.62, 70.49, 70.40, 70.32, 70.24, 69.56, 68.61, 62.14, 58.94, 52.22, 45.41, 40.37, 16.34, 12.49. MALDI-TOF MS: calcd for M + H^+ $C_{124}H_{177}Fe_2N_6O_{34}S_2$ 2471.05, found 2471.00.

Preparation of Fe(II)−MGD Complex and EPR Analysis. *N*-(Dithiocarbamoyl)-*N*-methyl-D-glucamine, sodium salt (MGD) is commercially available from Dojindo Molecular Technologies, Inc. The Fe(II)−MGD complex was prepared by mixing 1 mL of MGD solution (50 mM) with 8.8 mL of buffer solution (pH = 7.4) and then adding 200 μL of $FeSO_4$ solution (50 mM). Please note that any dissolved oxygen in the water or buffer should be purged by nitrogen gas bubbling for at least 30 min. A sample containing 0.4 mM **2P-M** and 10 mM Fe−MGD in distilled water was introduced to a quartz flat cuvette cell. EPR spectra were recorded after UV light irradiation (365 nm from UV lamp with optical intensity of ~5 mW/cm^2) for 10 min or IR light irradiation (775 nm from a Ti/sapphire laser with an average power at the sample: ~0.8 W/cm^2) for 20 min. The spectrometer settings were modulation frequency, 100 kHz; modulation amplitude, 2.024 G; scan time, 3 min; microwave power, 15.9 mW; and microwave frequency, 9.77 GHz.

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Two-Photon Excited Fluorescence. As an excitation source, a Ti/sapphire laser oscillator/amplifier system (CPA-2010 from Clark-MXR) was used, which generated pulses at ~775 nm with a pulse duration of ~165 fs at a repetition rate of 1 kHz. The laser beam was focused by an $f = 20$ cm lens, and the cell with sample solution was placed at a distance of ~22 cm from the focusing lens. The fluorescence of the sample was collected at 90° from the excitation light by using a HoloSpec CCD-array spectrometer in conjunction with a fiber coupler head.

Two-Photon Absorption Spectrum. A mode-locked Ti/sapphire laser (Mira from Coherrent), pumped by a DPSS laser (Verdi from Coherent), was used as the excitation source and generated pulses (~120 fs duration, 76 MHz repetition rate, ~2 nJ/pulse) from 730 to 880 nm. A spectrum analyzer (IST-rees) was used to monitor the excitation wavelength. The laser beam was focused into the center of the sample solution using a 5 cm focal length lens, and the fluorescence spectra were acquired using a Jobin-Yvon Fluorolog-3 spectrometer. The total fluorescence intensity collected for compound **2P-I** (10 μM in water) and the standard (Rhodamine 6G, 110 μM in methanol) at each excitation wavelength was calculated as the area under the collected fluorescence spectrum. The two-photon cross-section of Rhodamine 6G (110 μM solution in methanol) reported by Albota et al.[27] was used as a standard for calculating the two-photon cross section of compound **2P-I**.

Cell Viability Assay (UV Light Irradiation). For studying cell-viability, 24-well plates were inoculated with Hela cells (7.5×10^5 cells/well) and incubated overnight. The medium was removed; the wells were rinsed three times using sterile PBS, and 2 mL of fresh medium was added to each well. 50 μL of compound **2P-M** with predetermined concentration (0.12 mM/L) was added to each designated well. The plates were then returned to the incubator for two hours. Next, the plates were rinsed three times with sterile PBS. Two mL of the fresh medium was added and the plates were immediately exposed to a 365 nm light source for 40 min each. The optical intensity at the cell level was ~5 mW/cm^2. The plates were returned to the incubator overnight. Cell viability was estimated by means of the colorimetric MTT assay. In the MTT assay, the absorbance of formazan (produced by the cleavage of MTT by dehydrogenases in living cells) at 570 nm is directly proportional to the number of live cells.

Cell Viability (IR Light Irradiation). The Cos-7 (African green monkey kidney cell lines) or Hela (cervical adenocarcinoma cell lines) cells were dispensed into a 96-well flat-bottomed microtiter plate (Nunc) at a concentration of 10000/well and allowed to attach overnight using for growing MEMalpha and 10% FBS. The medium was removed; the wells were rinsed three times using sterile PBS, and 100 μL of fresh medium was added to each well. 0–10 μL of compound **2P-M** with predetermined concentration (0.4 mM/L) were added to designated wells. The plates were then returned to the incubator for three hours. Next, the plates were rinsed three times with sterile PBS. 0.3 mL of the fresh medium was added and the plates were immediately exposed for 5 min each to a 775 nm laser source from the Ti/sapphire laser oscillator/amplifier system (CPA-2010 from Clark-MXR). The generated pulses have pulse duration of ~165 fs at a repetition rate of 1 kHz. No focusing lens was used for the light irradiation and the average power at the cell level was ~0.8 W/cm^2. The plates were returned to the incubator overnight. Cell viability was assessed by the Cell Titer-Glo luminescent cell viability assay (Promega Corporation, Madison, WI). This assay is a homogeneous method to determining the number of viable cells in culture based on quantity of the ATP present, which signals the presence of metabolically active cells. In brief, Cell Titer reagent was added to the cells after the experiments and samples were mixed for 2 min and allowed to incubate for 10 min at room temperature. Luminescence was measured using a microplate luminometer (Synergy HT microplate reader Bio-Tek) and data were expressed as a percentage of the control. Tests were performed at least in quadruplicate. Each point represents the mean ± SD (bars) of replicates from one representative experiment.

Acknowledgment. This work was supported in part by a grant from the Chemistry and Life Sciences Directorate of the Air Force Office of Scientific Research and in part by the John R. Oishei Foundation. Partial support from the center of Excellence in Bioinformatics and Life Sciences at the University at Buffalo is also acknowledged. We thank Dr. Dinesh Sukumaran for his help in measuring EPR spectra.

MP700117S