

Platinum-Doped Dendritic Structure as a Phosphorescent Label for Bacteria in Two-Photon Excitation Microscopy

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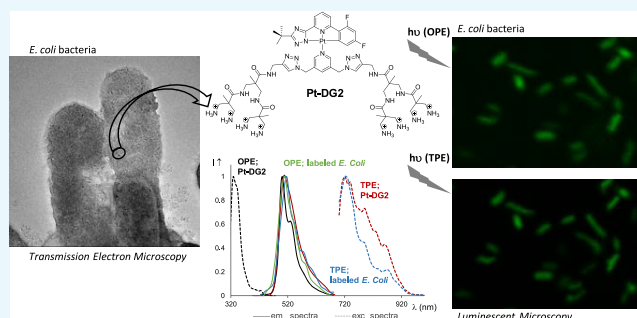
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Supporting Information

ABSTRACT: Herein, we present a water-soluble dendritic Pt(II) complex as a phosphorescent label for bacterial cells. The dendritic moiety endows the Pt(II) complex with unique properties such as water solubility, shielding from quenching by dioxygen, and binding to bacterial surfaces. The new biosensor was employed for two-photon excitation microscopy, and the binding was confirmed by electron microscopy, which demonstrates that such hybrid arrays can provide orthogonal yet complementary readouts.



INTRODUCTION

The luminescence properties of platinum(II) complexes have attracted considerable attention in the last decade, due to their potential application in the field of bioimaging.¹ The versatility of Pt(II) complexes lies in the tunability of their luminescence to blue, green, red, or white by tailored ligand design,^{2–5} their photostability, and their significant Stokes shift.⁶ Moreover, the ability of Pt(II) complexes to emit upon two-photon excitation (TPE) affords the possibility of in vivo imaging in tissues.⁷ However, despite these qualities, their application in the field of bioimaging is still limited due to solubility and quenching phenomena in aqueous environments.

Pt(II) complexes possess square-planar coordination geometries that favor strong axial interactions. Therefore, most of them interact face-to-face, stacking into aggregates. This in turn leads to broad red-shifted emission bands. The excimeric luminescence from aggregates is attributed to metal-metal-to-ligand charge transfer states (³MMLCT), whereas monomeric species emit from triplet ligand-centered (³LC) excited states with sizeable metal-to-ligand charge transfer (³MLCT) character.⁸ This metal-perturbed ligand-centered (³MP-LC) luminescence is usually quenched by triplet dioxygen in solution (³O₂), leading to shortened emission lifetimes, reduced luminescence intensities and reactive oxygen species (ROS) such as singlet dioxygen (¹O₂).⁹ Even if useful for

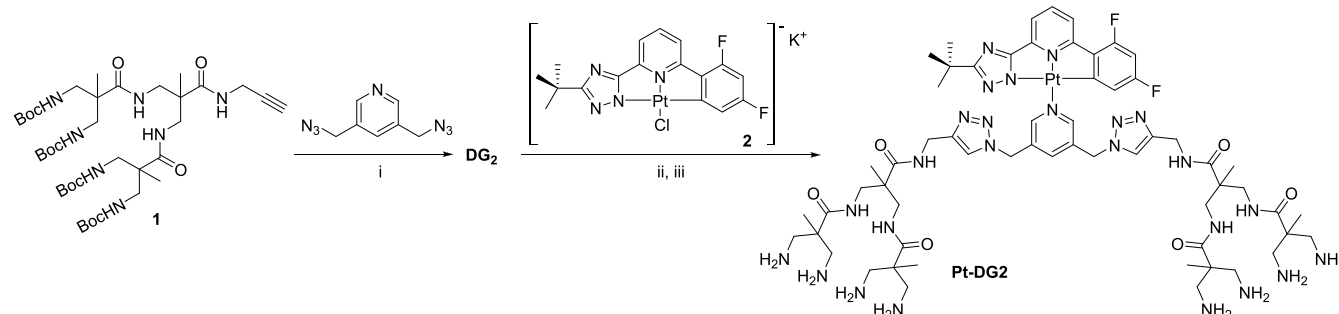
photodynamic therapy (PDT), the photoproduction of ROS can be detrimental for optical imaging, where high luminescence intensities are necessitated and cytotoxicity is to be avoided.¹⁰ Thus, it is necessary to suppress diffusional quenching by shielding the luminophoric complex. Adequate ligands surrounding the metal center can control the alignment of the core and tune π – π staking in solution or in solid media, where the formation of aggregates can be favored or hindered on demand.^{11,12}

We have recently reported on compact clusters of Pt(II) complexes adsorbed at the interface of bovine serum albumin, which are intrinsically shielded from molecular oxygen that allows a time-gated separation of their phosphorescence from background autofluorescence. While these clusters can even act as donors in energy transfer processes, the free monomeric species in solution are nonemissive.¹³ Moreover, we have shown that self-assembled micelles based on tailored copolymers doped with Pt(II) complexes can be tracked by optical and electron microscopy, due to their oxygen-insensitive luminescence and contrast enabled by the high atomic number. The resulting dual readout was employed to

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Scheme 1. Synthesis of the Pt-DG2 Complex^a

^aSynthesis of the dendritic Pt(II) complex: (i) copper(II) sulfate and L(+)-ascorbic acid sodium salt in *tert*-butanol/water (1:2); (ii) THF, 80 °C, overnight; (iii) trifluoroacetic acid, THF, overnight.

track their uptake and localization in eukaryotic cells with high spatiotemporal resolution.¹⁴

Solubility in aqueous media and shielding of the luminophores from diffusional quenching are two key aspects to be tackled. In this sense, dendrimers (dendrons) constitute appealing tools. They are highly branched, three-dimensional macromolecules, where the size, structure, and surface functionality are controllable. A few dendritic structures have been able to encapsulate and to isolate the emitting core Pt(II) complexes, thus decreasing intermolecular interaction in organic solvents.^{15–17}

We reasoned that the use of alkyl chain-based dendritic structures to protect the emitting core of a Pt(II) complex should not affect its photophysical properties, due to the nonconjugated nature of the encapsulating array, while the functional groups at the periphery would control the solubility. In this work, we present a concept combining the unique excited-state characteristics of Pt(II) complexes with the features of water-soluble dendrimers. We demonstrate that an amino-terminated amide–alkyl dendritic structure can effectively avoid aggregation while protecting the emitting core from quenching by molecular oxygen. Additionally, as a proof of concept, we demonstrate the ability of such a luminophore to act as a biosensor, labeling the cells with a green phosphorescence that can be used for two-photon excitation microscopy.

RESULTS AND DISCUSSION

Synthesis and Characterization. In the design of the amino-terminal amide–alkyl dendritic-Pt, we chose a BAPAD type structure as a template,¹⁸ and a 3,4-disubstituted pyridine as an anchoring point for the Pt(II) complex.¹¹ For the synthesis of the dendritic structure (DG2, Scheme 1, Supporting Information (SI)), a Boc-protected generation 2 dendron with an alkyne moiety in the focal point (1, Scheme 1, SI) was prepared and coupled with 3,5-bis(azidomethyl)pyridine via a copper-catalyzed azide–alkyne “click” cycloaddition (CuAAC).¹⁹ Complex 2 (Scheme 1) was synthesized as previously described.¹¹ The Pt-containing dendritic structure was obtained by a convenient exchange reaction of the chlorido ligand. After deprotection of the terminal amino groups, Pt-DG2 was obtained with a 13% yield. The resulting complex is soluble in aqueous media, due to the eight amino groups of the structure. These amino groups not only ensure solubility in aqueous media, but also modulate the biological activity of the molecule (such as promoting the adhesion to

bacteria) and can be conjugated to a variety of structures with relevant biological properties.²⁰

Characterization by NMR spectroscopy (in D₂O) confirmed the presence of the dendritic complex (see the SI); broad signals between 0 and 4 ppm are characteristic of the dendritic moiety together with two singlets at 4.44 and 5.69 ppm, corresponding to the methylene groups adjacent to the triazole ring. The presence of the Pt core is confirmed by the signal between 7.5 and 9 ppm. However, no mass spectrum was obtained, probably due to the lability of the Pt–N bond of the ancillary ligand.

Diffusion-ordered spectroscopy (DOSY) experiments were carried out observing a monoexponential decay of the signal (at a concentration of 10^{−3} M, Figure 1). This results in a linear Stejskal–Tanner plot, showing that diffusion of a monodisperse molecule is monitored, which excludes any aggregation (Figure 1).^{21,22}

Photophysical Characterization. The steady-state and time-resolved luminescence spectroscopy studies of Pt-DG2 in

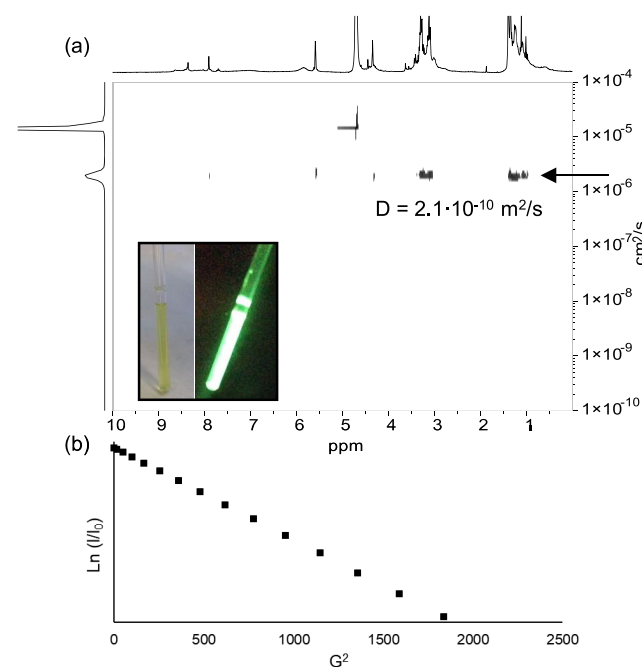


Figure 1. (a) DOSY NMR experiment for Pt-DG2; inset: picture of the NMR tube under visible light (left) and excitation at 365 nm (right); (b) Stejskal–Tanner plot.

water showed an absorption band at around 250 nm, which can be assigned to $\pi-\pi^*$ excitations, as well as a broad band around 350 nm that can be ascribed to a transition with mixed MLCT/LC character (Figure S45, SI).¹¹ In agreement with the results observed in the DOSY experiments, no significant evidence for ground state aggregation is observed.

The green luminescence exhibited by the complex (Figure 2, Table 1) is partially quenched by molecular oxygen. In fact,

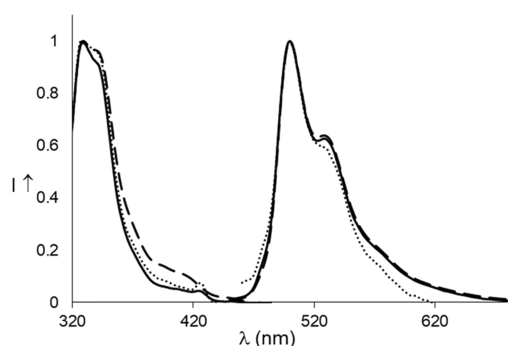


Figure 2. Excitation (left, $\lambda_{\text{em}} = 500$ nm) and emission spectra (right, $\lambda_{\text{exc}} = 350$ nm) of **Pt-DG2**, in aerated aqueous solutions at 10^{-6} M (dotted line), 10^{-5} M (solid line), and 10^{-4} M (dashed line).

both the photoluminescence quantum yields and the amplitude-weighted average lifetimes are enhanced by a factor of roughly 3 in deoxygenated samples (Table 1). The biexponential decay can be attributed to the ensemble of conformations that lead to different decay rates in the dendritic environment, despite being originated from the same excited state. Complex environments can lead to such an observation, as discussed in the literature.²³ Even though aggregation phenomenon could also account for this observation, the profile of the emission band corresponds to a monomeric species in a broad concentration range.

Although the formation of a small amount of aggregates cannot be completely ruled out when increasing the concentration (up to 10^{-4} M), no significant contribution of aggregates is observed at around 650 nm (Figures 2 and S46).

Pt-DG2 is luminescent in aqueous environments without the need for complex carriers, such as proteins, micelles, or polymers (Figure S47, SI). When comparing these results with similar Pt(II) complexes possessing the same $\hat{\text{C}}\hat{\text{N}}\hat{\text{N}}$ lumino-phore (derived from **2**, Scheme 1), a clear influence of the dendritic moiety can be observed,¹¹ as both the quantum yield and the lifetime appear enhanced in fluid environments but without traces of excimeric emission. When the ancillary ligand is a 4-substituted pyridine, a broad red-shifted emission band (around 600 nm) appears at higher concentrations, which can be associated with stacked species. The wavelength of such emission (normally from ³MLCT states) depends on the bulkiness of the ancillary ligand and its tilt angle with respect to the coordination plane.¹¹ Similar Pt(II) complexes have also

been modified using a 4-substituted pyridine with tetraethylene glycol chains as the ancillary ligand. In that case, solubility in aqueous solution was partially achieved, observing ground state aggregation at concentrations above 10^{-4} M. Above this concentration, emission of the aggregates was observed at 580 nm as well, and luminescence of the monomeric species was observed at any concentration at room temperature.²⁴

The bulky dendritic ligand **DG2** prevents intermolecular interactions even in aqueous media. Only absorption and emission due to the monomeric species are observed in aerated aqueous solutions of **Pt-DG2**, showing higher luminescence quantum yields than similar complexes.^{11,24} The long lifetimes observed for **Pt-DG2** indicate that the dendritic structure protects the platinum core against the environment, partially avoiding quenching of the luminescence by molecular oxygen. This is a key feature for imaging, as it suppresses at least partially the formation of cytotoxic reactive oxygen species while retaining the phosphorescence in oxygenated samples. The residual sensitivity to molecular dioxygen could be used to highlight hypoperfused or anoxic samples and tissues, as a prolongation of the excited-state lifetime could be monitored.

Excitation and emission spectra acquired by two-photon excitation (TPE) conditions were also recorded on a confocal optical microscope equipped with a high-performance laser and a spectrograph (Figure S52, SI). Taking into account the difference in resolution, the emission observed at 405 nm coincides with the one measured on the photoluminescence spectrometer in the one-photon excitation regime. When TPE spectra were recorded for related Pt(II) complexes where the ancillary ligand is $\text{P}(\text{Ph})_3$, no appreciable emission was observed in THF solution at 10^{-5} M (Figure S51, SI). In this particular case, even concentrations up to 10^{-4} M only provided an extremely weak signal, where the contribution of the aggregates is detectable. In all cases, the two-photon nature of the observed phenomenon was confirmed by the quadratic dependence of the emission intensity on the laser power (Figures S51 and S52, SI).

Pt-DG2 presents therefore the possibility of combining one- and two-photon excitation (OPE or TPE, respectively), opening the way for integrating multiple excitation channels to observe its luminescence. This is, to the best of our knowledge, the first example of a Pt(II) complex with this potential in aqueous media.

Labeling of *E. coli* (Gram-Negative) and *P. subtilis* (Gram-Positive) Bacteria with **Pt-DG2.** To evaluate the use of **Pt-DG2** as a multichannel luminescent marker for biological samples, two *E. coli* and *P. subtilis* bacterial strains were employed as a model targets. The amino-terminal groups of **Pt-DG2** provide an effective binder to the Gram-negative and Gram-positive bacterial surface, owing to their capacity for hydrogen bonds and electrostatic interaction.²⁰ After incubation (2 h) with a 10^{-4} M solution of the complex, **Pt-DG2** effectively labeled both *E. coli* and *P. subtilis* bacteria, as can be

Table 1. Photophysical Data of **Pt-DG2**^a

air-equilibrated		deoxygenated	
$\phi_L \pm 0.02$	$\tau \pm 0.01/\mu\text{s}$	$\phi_L \pm 0.02$	$\tau \pm 0.01/\mu\text{s}$
0.05	9.8 (28%); 3.1 (78%); [5.0]	0.12	19.0 (57%); 11.1 (43%); [15.5]

^a ϕ_L quantum yield and lifetime ($\lambda_{\text{em}} = 500$ nm) of aqueous solutions of **Pt-DG2**, 10^{-5} M (fractional amplitudes in parentheses, amplitude-weighted average lifetimes in brackets).

seen in the optical micrographs (Figures 3, S54, and S55 in the SI).

An intense green luminescence can be observed from the outer surface of the bacteria, confirming the adhesion of the complex to the bacterial wall. This characteristic emission can be observed under both OPE and TPE conditions. This is a key point when performing imaging experiments of biological

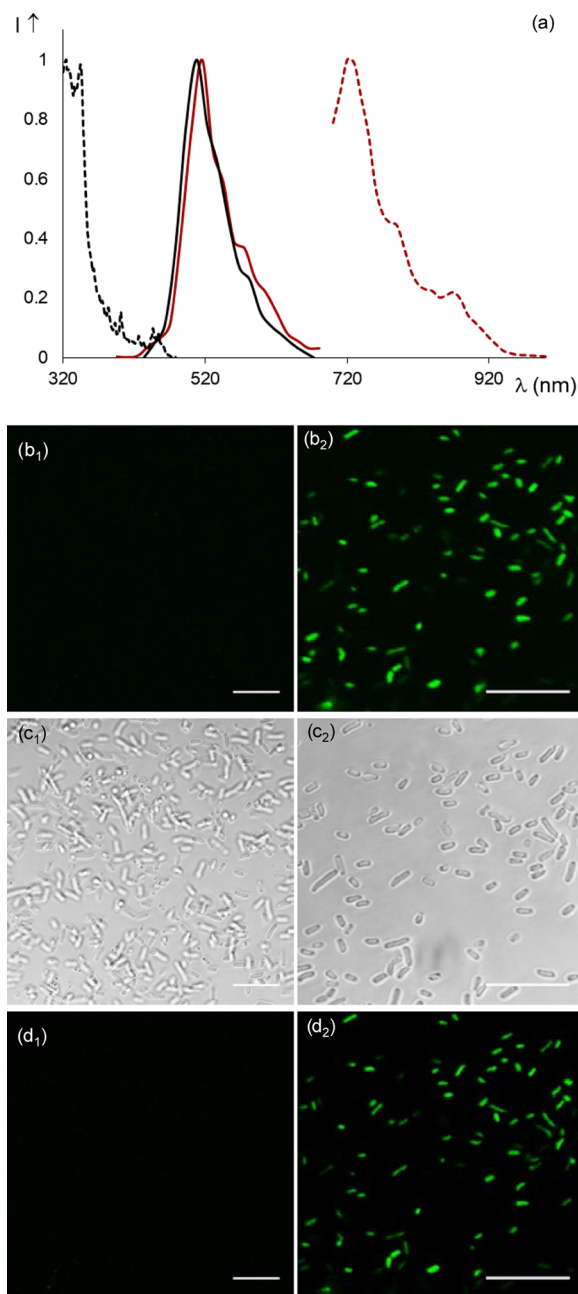


Figure 3. (a) Excitation (dotted line) and emission (solid line) spectra of *E. coli* incubated for 2 h with Pt-DG2 upon one-photon (black) or two-photon (red) excitation (405 and 720 nm, respectively), in aqueous solutions. Confocal micrographs of *E. coli* incubated for 2 h with (right row; b_2 , c_2 , and d_2) or without (left row; b_1 , c_1 , and d_1) Pt-DG2: (b_1 , b_2) recorded emission with one-photon excitation ($\lambda_{\text{exc}} = 405$ nm; collected through 500–600 nm); (c_1 , c_2) bright-field images; (d_1 , d_2) recorded emission with two-photon excitation ($\lambda_{\text{exc}} = 720$ nm; collected through 500–550 nm). Scale bars: 5 μm .

samples, which is relevant to distinguish the intrinsic emission of the luminescent probe from sample autofluorescence and background noise.²⁵ In untreated bacteria, an extremely weak autofluorescence is observed under OPE. However, no autofluorescence is observed under TPE conditions (capture settings and image processing are identical). Moreover, the emission and excitation spectra of the stained bacteria resemble those of Pt-DG2 in both OPE and TPE conditions (Figures 2, 3a and S52, SI). The longer excited-state lifetime observed for stained bacteria (10 μs) also indicates the presence of the monomeric species and the protection of the Pt(II) core from quenching processes.

The dynamics of bacterial growth was monitored in liquid LB medium originally inoculated with 4×10^6 *E. coli* and *P. subtilis* and incubated in the presence (10 or 100 μM) or absence of Pt-DG2. No significant *E. coli* growth delay was recorded with increasing Pt-DG2 concentration from 10 to 100 μM , but *P. subtilis* growth was significantly reduced at 100 μM Pt-DG2. (Figure S56 in the SI).

Transmission electron microscopy (TEM) images were also acquired, where energy dispersive X-ray (EDX) analysis confirms the presence of platinum (Figures 4 and S57 SI). Although no measurable interaction between the metal centers is observed in solution, TEM images of incubated cells show high contrast clusters that are not observed in untreated bacteria (red arrow in Figure 4b,c). Such Pt clusters provide a possibility of tracking heavy atoms by means of electron microscopy.

CONCLUSIONS

By combining the characteristics of dendritic structures with the photophysical properties of Pt(II) complexes, we obtained a unique luminescent marker soluble in aqueous media. The dendritic moiety, with well-defined structure and three-dimensional geometry, possesses the ability to encapsulate the emitting core and create a specific site-shielded nano-environment. In this way, the complex constitutes a triplet emitter whose photophysical properties are perfectly stable in biological media without the need for a carrier. The approach to exploiting the luminescence properties of Pt(II) complexes in bioimaging has been demonstrated using both OPE and TPE conditions. Additionally, the array has proven to be traceable by electron microscopy and acts as a probe providing dual orthogonal readouts.

EXPERIMENTAL SECTION

Chemicals and Materials. All reactions were performed using commercially available reagents and solvents as received from the manufacturer, without further purification. Unless otherwise stated, all reactions were performed in air. Column chromatography and thin layer chromatography (TLC) were performed on silica gel 60 (0.040–0.063 mm) using UV light and/or stains to visualize the products. SephadexTM G-10 prepacked columns were used to purify the final compound.

Instruments and Characterization. ^1H and ^{13}C NMR spectra were measured in the indicated deuterated solvent at 25 $^\circ\text{C}$ on a Bruker Ascend 400 MHz spectrometer and on a Bruker Ascend 600 MHz spectrometer. Chemical shifts (δ) are given in parts per million with respect to the residual protonated solvent employed as the internal standard (CDCl_3 δ 7.26, $\text{DMSO}-d_6$ δ 2.50, D_2O δ 4.79, $\text{MeOD}-d_4$ δ 3.31). Data are reported as follows: chemical shift, multiplicity,

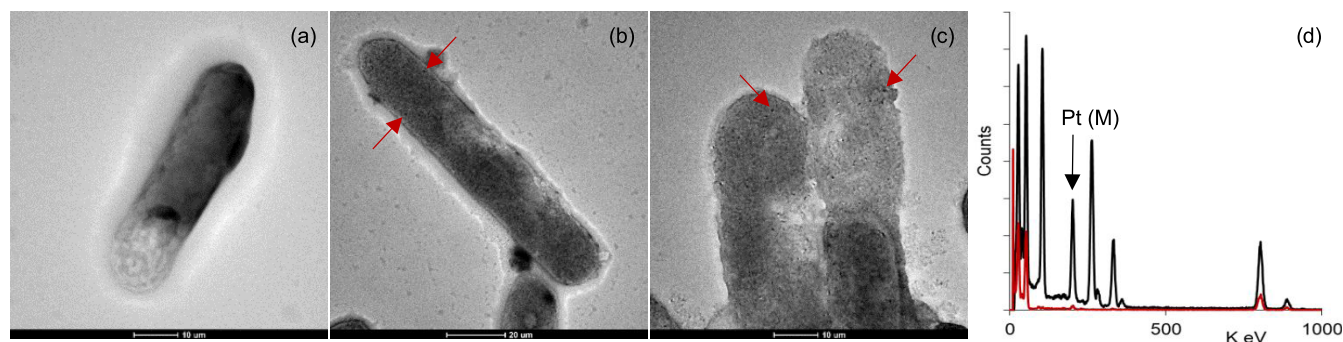


Figure 4. TEM images of (a) *E. coli* and (b, c) *E. coli* incubated (2 h) with Pt-DG2 (red arrows highlight some of the Pt clusters observed). Scale bars: 20 and 10 μm . (d) EDX spectra obtained from incubated *E. coli* (black) and nonincubated *E. coli* (red).

coupling constants (Hz), and integration. Infrared (IR) spectra were recorded using a Jasco FT/IR-4100 spectrophotometer. IR spectra were obtained at ambient temperature. HRMS (electrospray ionization time-of-flight (ESI-TOF) mass spectra (MS)) were performed on a High-Resolution Mass Spectrometer Orbitrap, Q-Exactive (Thermo Fisher Scientific, Waltham, MA). Hydrogenolysis reactions were carried out under a hydrogen atmosphere (50 bar) using a Mini-Reactor from Erie-Autoclave Engineers. Luminescence measurements were performed using an Edinburgh Instruments FLS920 spectrometer equipped with a 450 W Xenon lamp (Xe900) as a continuous excitation source for stationary state measurements and Picoquant PLS-450 and PLS-500 pulsed light emitting diodes (LEDs) as pulsed excitation sources for time-resolved measurements. Phosphorescence measurements were performed using a detector system with an R928P photomultiplier-based delay generator. Lifetime analysis was also performed using the commercial FluoFit software. The quality of the fit was assessed by minimizing the reduced chi-squared function (χ^2) and visual inspection of the weighted residuals and their autocorrelation. Luminescence lifetimes were recorded in TCSPC mode by a PicoHarp 300 (minimum base resolution 4 ps) on a FluoTime300 spectrometer from PicoQuant equipped with a 300 W ozone-free Xe lamp (250–900 nm), a 10 W Xe flash-lamp (250–900 nm, pulse width <10 μs) with repetition rates of 0.1–300 Hz, an excitation monochromator (Czerny-Turner 2.7 nm/mm dispersion, 1200 grooves/mm, blazed at 300 nm), diode lasers (pulse width <80 ps) operated by a computer-controlled laser driver PDL-820 (repetition rate up to 80 MHz, burst mode for slow and weak decays), two emission monochromators (Czerny-Turner, selectable gratings blazed at 500 nm with 2.7 nm/mm dispersion and 1200 grooves/mm, or blazed at 1250 nm with 5.4 nm/mm dispersion and 600 grooves/mm), Glan-Thompson polarizers for excitation (Xe-lamps) and emission, a Peltier-thermostated sample holder from Quantum Northwest (−40 to 105 $^{\circ}\text{C}$), and two detectors, namely a PMA Hybrid 40 (transit time spread FWHM <120 ps, 300–720 nm) and a R5509-42 NIR-photomultiplier tube (transit time spread FWHM 1.5 ns, 300–1400 nm) with external cooling (−80 $^{\circ}\text{C}$) from Hamamatsu.

Synthetic Methods. Experimental procedures for the synthesis of compound 1 and 3,5-bis(azidomethyl)pyridine can be found in the Supporting Information (Scheme S1).

Synthesis of DG2. 3,5-Bis(azidomethyl)pyridine (30 mg, 0.16 mmol, see SI), compound 1 (250 mg, 0.31 mmol), copper(II) sulfate 5-hydrate (4 mg, 0.016 mmol), and L(+)-ascorbic acid sodium salt (13 mg, 0.064 mmol) were dissolved

in *tert*-butanol/water 1:2 (9 mL). The mixture was stirred at room temperature for one week. Afterward, the solvent was removed using rotary evaporation. NH_4OH (50 mL) and dichloromethane (50 mL) were added and the phases were separated. The aqueous phase was extracted with CH_2Cl_2 (3 $\times$ 30 mL). The combined organic layers were washed with NH_4OH /brine 1:1 (3 $\times$ 80 mL). The organic layer was dried over MgSO_4 , and the solvent was removed by rotary evaporation. The product was purified by precipitation in hexane to obtain DG2 (234 mg, 83%) as a white solid. ^1H NMR (400 MHz, $\text{DMSO}-d_6$) δ : 8.54–7.97 (m, 5 H), 5.61 (s, 4 H), 4.33–4.25 (m, 4 H), 3.61–3.45 (m, 4 H), 3.20–2.83 (m, 20 H), 1.35 (s, 72 H), 1.09–0.91 (m, 18 H). ^{13}C NMR (100 MHz, $\text{DMSO}-d_6$) δ : 174.8, 174.6, 156.1, 149.0, 145.5, 135.6, 131.7, 123.1, 77.9, 50.0, 47.9, 46.7, 44.6, 42.4, 34.8, 28.1, 19.2, 18.5. HRMS calcd for $\text{C}_{83}\text{H}_{141}\text{N}_{21}\text{O}_{22} \text{Na}^+$ 1807.0474 [$\text{M} + \text{Na}$] $^+$, found 1807.0452.

Synthesis of Pt-DG2. DG2 (50 mg, 0.028 mmol) and Pt(II) complex 2¹¹ (16 mg, 0.028 mmol) were dissolved in THF (10 mL) and heated at reflux overnight. The solvent was removed under vacuum, and the residue was dissolved in ethyl acetate (30 mL) and washed with water (3 $\times$ 30 mL). The organic phase was dried over MgSO_4 , and the solvent was removed. The obtained compound was dissolved in dry THF (5 mL) and the solution was cooled in an ice-water bath. TFA (2 mL) was added dropwise, and the mixture was stirred overnight. Afterward, the solvent was evaporated under vacuum. The residue was dissolved in ethyl acetate (10 mL) and extracted with water (3 $\times$ 10 mL). The aqueous phase was lyophilized, and the remains were purified by a sephadex column to obtain Pt-DG2 as ammonium salt (9 mg, 13%). ^1H NMR (600 MHz, D_2O) δ : 9.00–7.68 (m, 10 H), 5.69 (s, 4 H), 4.44 (s, 4 H), 3.80–2.95 (m, 24 H), 1.65–0.99 (m, 27 H). ^{13}C NMR (100 MHz, D_2O) δ : 175.3, 172.4, 162.3, 161.9, 150.1, 148.4, 147.4, 144.04, 143.97, 136.1, 130.8, 123.57, 123.55, 119.9, 117.0, 114.1, 111.2, 107.9, 50.1, 43.6, 43.4, 43.3, 42.8, 42.7, 33.8, 28.3, 16.8, 16.4.

DOSY Nuclear Magnetic Resonance (NMR) Experiment for Pt-DG2. The sample was prepared in deuterium oxide at a concentration of 1 mM. The experiments have been performed on a Bruker Ascend 400 MHz spectrometer, equipped with a 5 mm BBFOPLUS probe with a 2H “lock” channel and z gradient. The spectrometer is also equipped with a control temperature unit prepared to work at temperatures ranging from 0 to +50 $^{\circ}\text{C}$. Gradient strength was calibrated by measuring the diffusion rate of pure water of residual protons in D_2O . Experiments were conducted at 300 K. The samples were equilibrated for no fewer than 15 min. To determine the

diffusion rates, a two-dimensional sequence using double stimulated echo for convection compensation and a LED using bipolar gradient pulses for diffusion were used. A diffusion constant of $2.1 \times 10^{-10} \text{ m}^2/\text{s}$ was observed.

Two-Photon Excitation Experiments. Excitation and emission scans were performed using a Leica SP5 MP confocal microscope equipped with a Spectraphysics MaiTai HP pulse IR laser for multiphoton excitation and an HCX PL APO lambda blue 63 $\times$ NA 1.40 oil immersion objective lens. For excitation scans, fluorescence was measured between 500 and 600 nm using an internal PMT detector with excitation varying from 700 to 1040 nm at 10 nm intervals. For emission scans, samples were excited at 720 nm and emissions were measured using internal spectral PMT detectors as a 30 nm window moving from 400 to 700 nm in 15 nm intervals (20 measurements). TPA cross-sections were determined by the TPEF method.²⁶ It is assumed that the quantum efficiencies after two-photon excitation are the same as those after one-photon excitation. The TPA cross-sections (δ) were obtained by calibration against rhodamine B with a known δ value in MeOH solution. Then, the TPA cross-section δ values were calculated on the basis of the following expression:

$\delta_s = \delta_r \frac{C_r \eta_r \phi_r F_s}{C_s \eta_s \phi_s F_r}$, where δ is the TPA cross-section, C and η are the concentration and refractive index of the sample solution, and F is the integrated area under the TPEF spectrum. Pt-DG2 shows TPA cross-sections δ of about 3.90 GM [1 GM (Goepfert–Mayer unit) = $10^{-50} \text{ cm}^4 \text{ s molecule}^{-1} \text{ photon}^{-1}$] (710 nm).

Luminescence Microscopy Experiments with *E. coli* (Gram-Negative) and *P. subtilis* (Gram-Positive) Bacteria Incubated with Pt-DG2. Both bacteria (*E. coli* and *P. subtilis*) were grown in 10 mL of LB Broth at 37 °C in a rocking incubator (18 h). Then, culture contents were split into four 15 mL vials, centrifuged (5000g, 5 min), and washed again in 5 mL of PBS. After an additional centrifugation (5000g, 5 min), bacterial pellets were either resuspended in 3 mL of PBS with Pt-DG2 (10^{-4} M dilution) or resuspended in 3 mL of PBS alone, followed by a 2 h incubation step in a rocking incubator (37 °C). Then, both samples were centrifuged (5000g, 5 min) and washed twice in 3 mL of PBS. Finally, each bacterial sample was resuspended in 100 μL of PBS. Bacterial cultures were analyzed using a Leica SP5 MP confocal microscope equipped with Spectraphysics MaiTai HP pulse IR laser for multiphoton excitation and an HCX PL APO lambda blue 63 $\times$ NA 1.40 oil immersion objective lens was used. Bright-field and confocal images were acquired using 405 nm excitation with emissions detected with a spectral PMT detector set to 500–600 nm. Multiphoton images were acquired sequentially with excitation at 720 nm and detection between 500 and 550 nm with an external HyD nondescanned detector.

Bactericidal Test. To examine the bactericidal effect of Pt-DG2 on Gram-negative and Gram-positive bacteria, approximately 4×10^6 colony-forming units of *E. coli* and *P. subtilis* were cultured on LB agar plates supplemented with 10 or 100 μM Pt-DG2. Pt-DG2-free LB plates cultured under the same conditions were used as controls. The plates were incubated for 24 h at 37 °C, and the number of colonies was recorded. Counts on the three plates corresponding to a particular sample were averaged. To examine the bacterial growth rate as well as to determine the growth curve in the presence of Pt-DG2, *E. coli* and *P. subtilis* were grown in liquid LB medium

supplemented with 10 or 100 μM . Growth rates and bacterial concentrations were determined by measuring the optical density (OD) at 600 nm every 1 h (OD of 0.1 corresponds to a concentration of 10^8 cells per cm^3) in a FLUOstar Omega de BMG Labtech device.

Transmission Electron Microscopy (TEM) Experiments. TEM and EDX measurements of bacteria were performed using a Thermo Fisher Tecnai G2 20 Twin microscope. For TEM measurements, a drop of a solution of bacteria in PBS was placed in a copper grid covered with a Formvar-Carbon film and was left to dry overnight. No contrast agent was used for these samples. The voltage used was 120 kV, and the beam of electrons was between 6.94 and 8.80 μA . The exposure time for image capture was 1 s. To obtain EDX data, a magnification between 200 and 240 kX was used, focusing the beam on the center of the bacteria. The spectral measurement was carried out in such a way that the death time (waiting time for the acquisition between counts) was around 10–15%, with a total counting duration of 50 s.

■ ASSOCIATED CONTENT

■ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.9b00639.

Experimental procedures and data for related compounds, NMR spectra, infrared (IR) spectra, time-resolved luminescence decay of Pt-DG2, and two-photon excitation experiments (PDF)

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Author Contributions

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ABBREVIATIONS

PDT, photodynamic therapy; TLC, thin layer chromatography; UV, ultraviolet; HRMS, high-resolution mass spectrometry; TPA, two-photon absorption; TPEF, two-photon excitation fluorescence; PBS, phosphate-buffered saline; EDX, energy dispersive X-ray analysis

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