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Nitric Oxide Photogeneration from *trans*-Cr(cyclam)$(ONO)_2^+$ in a Reducing Environment. Activation of Soluble Guanylyl Cyclase and Arterial Vasorelaxation

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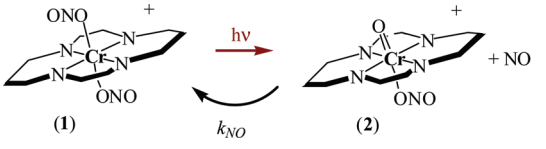
The chromium(III) nitrito complex *trans*-Cr(cyclam)$(ONO)_2^+$ (**1**) is a very promising photochemical precursor for nitric oxide delivery to physiological targets. Here, we demonstrate that visible wavelength excitation of **1** in solutions containing thiol reductants such as the biological antioxidant glutathione (GSH) leads to permanent reaction even under anaerobic conditions, resulting in high quantum yield NO release. The nitric oxide formed under such conditions is sufficient, even at μM concentrations of **1** and using a low-intensity light source, to activate the enzyme soluble guanylyl cyclase (sGC). We also demonstrate that photolysis of **1** in the nM concentration range with a portable blue LED leads to vasorelaxation of porcine coronary arterial rings, a process also attributed to the NO activation of sGC.

Introduction

Photochemical methods for the delivery of bioactive agents for therapeutic applications offer several key advantages over traditional drug delivery methods. Because photochemical delivery is modulated by the location and intensity of light irradiation, these methodologies allow for precise control over the target site as well as the timing and the dosage of the agent released.[1,2] Thus, we have a continuing interest in the synthesis of compounds capable of releasing nitric oxide (aka nitrogen monoxide, NO[a]) at specific biological target sites upon excitation with visible or near-infrared light.[3−12] This attention is motivated by the multitude of roles that NO plays in vasodilation[13−15] and in tumor growth or suppression[16−25] and by NO's potential application as a sensitizer in radiation oncology.[26−31] Using photolysis to initiate NO release from designed precursors would give the desired control, and, given the reactive nature of NO, the physiological effect of the release of this bioactive agent will be largely contained within the immediate irradiation location.

The chromium(III) dinitrito complex, *trans*-Cr(cyclam)-$(ONO)_2^+$ (**1**),[5,6] which is water-soluble and thermally stable at 37 °C, is a very promising photochemical NO precursor for potential therapeutic applications. Furthermore, **1** is photoactive throughout the visible region and displays a high quantum yield for reversible NO release, presumably via concomitant formation of the Cr(IV) oxo complex **2** (eq 1).[6] The back reaction is quite fast ($k_{NO} = \sim 3 \times 10^6\ M^{-1}\ s^{-1}$), but in aerated solution, net photoproduct formation is evident owing to oxidative trapping of **2**.[6] In the present work, we show that photolysis of **1** in the presence of the biological reductant, glutathione (GSH), leads to permanent photochemistry and NO release, *even in the absence of air*. We further demonstrate that **1** acts as an effective photochemical precursor of NO for the activation of soluble guanylyl cyclase (sGC) and for the vasorelaxation of porcine arterial rings in myography experiments.



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Experimental Section

Chemicals and Reagents. The sGC inhibitor, 1*H*-[1,2,4]-oxadiazolo[4,3-*a*]quinoxalin-1-one (ODQ), and vasoconstrictor 9,11-dideoxy-11α,9α-epoxy-methanoprostaglandin $F_{2\alpha}$ (U46619) were purchased from Sigma and kept frozen at −4 °C until use. Standard Krebs buffer (pH 7.4; composition (in mM): NaCl 119, KCl 4.7, $CaCl_2$ 2.5, $MgCl_2$ 1, $NaHCO_3$ 25, KH_2PO_4 1.2,

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[a] Abbreviations: BSA, bovine serum albumin; CFL, compact fluorescent light-bulb; cGMP, cyclic guanosine monophosphate; cyclam, 1,4,8,11-tetraazacyclotetradecane; DEA/NO, (*Z*)-1-(*N*,*N*-diethylamino)-diazen-1-ium-1,2-diolate; EDTA, ethylenediaminetetraacetic acid; GSH, glutathione; GTP, guanosine triphosphate; sGC, soluble guanylyl cyclase; IBMX, isobutylmethylxanthine; LED, light emitting diode; NO, nitric oxide; NOA, nitric oxide analyzer; ODQ, 1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one; U46619, 9,11-dideoxy-11α,9α-epoxy-methanoprostaglandin $F_{2\alpha}$.

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D-glucose 11) and high K^+ Krebs buffer (composition in mM: NaCl 119, KCl 60, $CaCl_2$ 2.5, $MgCl_2$ 1, $NaHCO_3$ 25, KH_2PO_4 1.2, D-glucose 11) were made up in sterile deionized water. GSH was purchased from Sigma-Aldrich and kept at 4 °C until use. All other chemicals were purchased from Sigma-Aldrich unless otherwise noted.

The *trans*-[Cr(cyclam)$(ONO)_2$]BF_4 salt was synthesized using a procedure developed by DeLeo et al.[32] Initial studies showed >99% purity by chromium analysis, and the UV−vis spectrum {λ_{max} in nm (ε in M^{-1} cm^{-1})}: 336 (267), 475 (40) is identical to that reported for this compound, also well characterized by X-ray crystallography and high resolution mass spectrometry.[32]

Photochemistry of 1 with Added GSH. Solution samples for photochemical studies were prepared in the dark in pH 7.4 phosphate buffer (15 mM) in a small Schlenk type flask to which a 1.0 cm path length quartz cuvette was attached. The concentration of *trans*-[Cr(cyclam)$(ONO)_2$]BF_4 was typically ∼400 μM, and such solutions were prepared both with and without added GSH (5 mM). The solutions were deaerated by a freeze−pump−thaw cycle under argon 3 times. Photolysis was then carried out using an optical train equipped with a high pressure mercury arc lamp excitation source and an interference filter to isolate the desired excitation wavelength (436 nm). The intensity of light was determined using ferrioxalate actinometry.[33] The solution spectra were periodically recorded on a HP 8452A diode array spectrophotometer to determine the extent of photochemical reaction.

Nitric Oxide Detection by Chemiluminescence. Photolysis-induced NO release from **1** was measured using a GE nitric oxide analyzer (model NOA-280i) for liquid samples. The NOA was calibrated by 10 μL injection of standard $NaNO_2$ solutions from 5 to 100 μM into a potassium iodide solution in glacial acetic acid. Samples of **1**, 100 μM to 1 mM in pH 7.4 aqueous 15 mM phosphate buffer solution, were photolyzed (λ_{irr} = 436 nm) for a known period of time (∼5−20 s) while the solution was being stirred and purged with the carrier gas, which was then passed into the NOA for analysis of the NO generated. For measurements in deaerated solutions, the carrier gas was purified helium, while for experiments in aerated solutions, the carrier gas was medical grade compressed air. In both cases, the carrier gas was first passed through a purge vessel filled with nanopure water. The actual bubbling site was maintained outside the volume of solution directly in the photolysis beam. Multiple experiments established a reproducible determination of the NO released. Analogous experiments were also carried out using sample solutions containing added GSH (5 mM).

sGC Activation. Bovine sGC was isolated and purified as described previously.[34] The procedure produced sGC that was BSA-free by Western blot analysis. Activation by NO was determined using a procedure modified from that previously reported.[35] Samples (200 μL) were prepared with sGC, 40 mM triethanolamine (TEA) buffer pH 7.4, 1 mM guanylyl triphosphate (GTP) substrate, 0.3 mM isobutylmethylxanthine (IBMX, phosphodiesterase inhibitor), 10 mM dithiothreitol (DTT), and 3 mM Mg^{2+}, and the appropriate concentration of *trans*-[Cr(cyclam)$(ONO)_2$]BF_4 (10 nM to 100 μM). Samples with sodium (*Z*)-l-(*N*,*N*-diethylamino)-diazen-l-ium-l,2-diolate (DEA/NO) as a reference NO source were also prepared (100 nM to 1 mM), as were solutions of **1** (100 μM) that had been exhaustively photolyzed. The latter solutions were used to examine whether the photoproducts from **1** other than NO itself might be playing a role in the activation of sGC. After preparation, samples were quickly placed on a heating block at 37 °C and either left in the dark or irradiated with a 14 W compact fluorescent lamp (N:Vision CFL, 3500 K, 120 V) at a distance of ∼12 cm. After 10 min, the enzymatic system was quenched by the addition of 1 M EDTA (to coordinate Mg^{2+}), and samples were frozen in the dark until analysis (overnight, or 2 h). Activation of purified sGC enzyme was measured by production of cGMP using a cGMP-specific ELISA assay (Parameter, R&D systems, or Amersham Biotrak (EIA) System). For analysis, the samples were thawed slowly in the dark and then diluted 100-fold and plated in 2 wells. The plate was prepared in the dark and incubated, washed, and developed as instructed. The optical density of each well was measured with a microplate reader. A calibration curve was constructed to correlate optical density with concentration of cGMP in the samples. From these data, the activation of sGC in the samples relative to the basal enzyme activity was obtained.

Vasorelaxation. Porcine hearts were obtained fresh from the local abattoir immediately after slaughter and kept in ice-cold Krebs buffer for transport. A strip (∼5 cm long) of the right descending coronary artery was removed carefully using fine scissors and forceps. All fat was removed from the artery prior to cutting it into ∼4 mm rings; the first and last sections of the artery were discarded as were any rings with branch points. The endothelium was removed by placing the arterial ring on forceps and gently scraping the intimal surface of the ring. Arterial rings were stored at 4 °C in Krebs buffer solution for up to 3 days. Arterial rings were mounted in a multichamber wire myograph (DMT; Aarhus, Denmark) with controller (Powerlab 4/30, AD Instruments, Oxfordshire, UK) in 10 mL Krebs buffer that was prewarmed to 37 °C and bubbled with 5% CO_2 /95% O_2. Arterial tension was sequentially increased over a period of 1 h, at which point the target passive tension was 15 mN. After that, the standard Krebs buffer was replaced with high potassium (60 mM) Krebs buffer to test smooth muscle function (3 × 3 min incubations interspersed with normal Krebs buffer incubations of 3 min each).

The arterial rings were then precontracted with the vasoconstrictor U46619 (100 nM). Full contraction occurs in ∼30 min. Any arterial ring that failed to generate >60 mN tension was not used further. The absence of the endothelium was confirmed by testing with a supramaximal concentration of the endothelium-dependent vasodilator, bradykinin (300 μM). The endothelium was deemed to be removed if relaxation failed to reach 5% after 3 min. The chambers were then washed with Krebs buffer, U46619 (100 nM) was added, and the arterial rings were given 10 min to contract fully again. A cumulative concentration response test to **1** (30 nM to 10 μM) was performed in three of the four wells, one with a blue LED array (ThorLabs) at a fixed distance of 10 cm from the top of the well (published intensity of the LED at this distance is 600 $\mu W/cm^2$ and the wavelength is centered at 470 nm), one covered in foil and one exposed to ambient light. It is important to note that some vasorelaxation was seen initially upon exposure of the arterial rings to the LED source in the absence of added **1**. This effect was attributed to the well-recognized phenomenon of photoactive *S*-nitrosothiols and/or other endogenous NO sources in the arterial tissue.[36−38] However, as seen previously,[36] the response was transient and recovered to baseline tension in ∼10 min, and additions of **1** were only made once direct photoactivation of the tissue had fully reversed. Subsequent exposures to light did not cause vasorelaxation in vessels without endothelium,[36] indicating that direct photorelaxation of vessels did not play a part in any subsequent responses. The fourth ring was treated with analogous concentrations of prephotolyzed **1**. The force on the arterial rings was recorded for 3 min after addition of each concentration. After the final drug addition, the light source was switched off and the exposed vessel allowed to recover to the baseline tension (∼10 min). Each arterial ring was then treated with the sGC inhibitor ODQ[39] (20 μM) for 15 min prior to repeating the experiment with cumulative additions of **1** in the presence and absence of light. In separate experiments, precontracted (U46619; 100 nM) endothelium-denuded porcine coronary artery rings were treated with 1 μM **1** under identical exposure conditions as described above (470 nm LED light). Exposure was maintained for >30 min in order to establish the duration of the response.

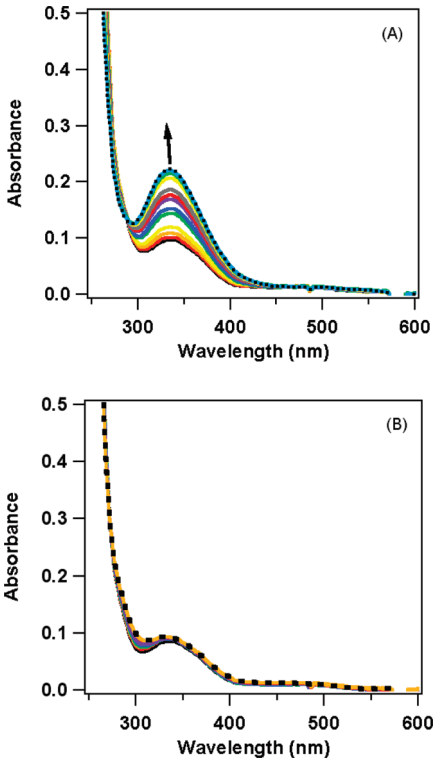


Figure 1. Photochemistry of *trans*-Cr(cyclam)$(ONO)_2^+$ (0.4 mM) in deaerated pH 7.4 phosphate buffer (15 mM) solution, 25 °C. The irradiation wavelength was 436 nm, and the absorption spectra shown were recorded initially approximately every 10 s, then approximately every minute. The final spectra (dotted lines) shown are obtained after approximately 1 h of photolysis. (A) Top: with added GSH (5 mM). (B) Bottom: without GSH.

Results and Discussion

Photochemistry of *trans*-Cr(cyclam)$(ONO)_2^+$ with Added GSH. As tumor tissues tend to be hypoxic,[40] an effective NO precursor for therapeutic applications would need to function in a relatively reducing and O_2 deficient environment. However, our earlier photochemical studies of **1** primarily involved aerated solutions, and we had observed little net photoreaction in deaerated media. This result was interpreted in terms of O_2 trapping of the Cr(IV) intermediate *trans*-Cr(cyclam)$(O)(ONO)^+$ (**2**) (eq **1**) to give Cr(V) products, which were identified. In the absence of such trapping, the back reaction of **2** with NO predominates, so that little net photochemistry resulted.[6] Thus, from the perspective of potential therapeutic applications of **1**, the requirement of oxidative trapping as a prerequisite for net NO photorelease would present problems under the hypoxic or anoxic conditions common to solid tumors. In this context, one might consider that the Cr(IV) intermediate **2** is also an oxidant and that such a species might therefore be subject to trapping by a reductant to give a Cr(III) complex that is unreactive with NO. To explore this possibility, we undertook a photochemical investigation of **1** in anoxic solutions containing GSH, a key biological antioxidant commonly present at 1−10 mM concentrations in various tissues.[41]

Figure 1 illustrates the spectral changes seen when deaerated solutions of **1** were subjected to 436 nm photolysis in the presence of 5 mM GSH and without any added GSH. Consistent with the earlier report,[6] the absorption changes were very small in anoxic solution when no additional reductant had been added (Figure 1B). However, the spectral changes were dramatically different in the presence of added glutathione (Figure 1A). Thus, we conclude that GSH does indeed trap transients generated in the primary photoreaction that would otherwise back-react to regenerate **1**. Furthermore, the photoreaction in the GSH containing solution appears to be quite clean with a single isosbestic point observed at ~290 nm. Notably, the spectral changes are not the same as would be seen for an aerated solution of **1** without GSH (see Supporting Information Figure S1), for which O_2 trapping was shown to give Cr(V) products.

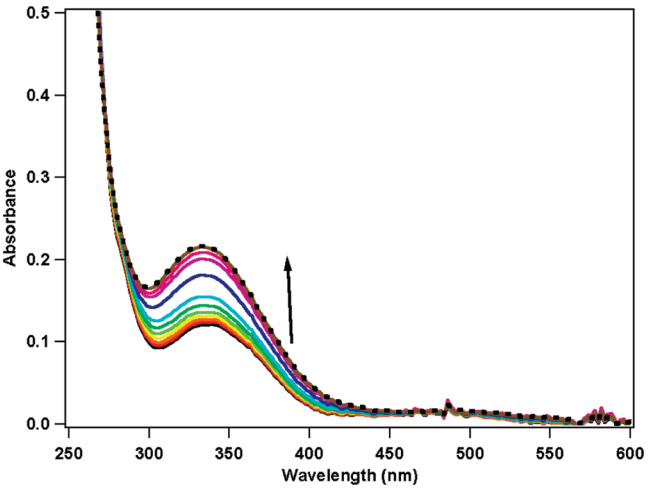
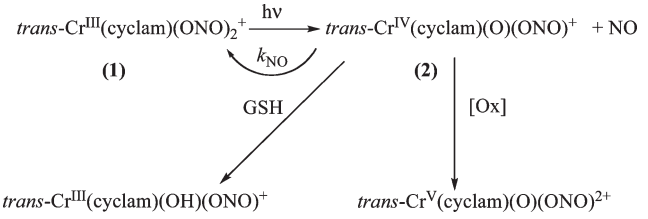


Figure 2. Changes in the optical spectrum of a solution of 1 (450 μM) in pH 7.4 15 mM phosphate buffer solution upon photolysis with 436 nm light at 25 °C in air. Spectra were taken approximately every minute for ~1 h to achieve complete photolysis (final spectrum is the dotted line).

Scheme 1



It should be noted that even in aerated solutions, the addition of GSH (5 mM) leads to photochemistry analogous to that seen in deaerated solutions with GSH. Figure 2 illustrates the photolysis-induced spectral changes for an *aerated* solution containing GSH. Notably, these are also considerably different from those seen in aerated solutions not containing GSH, thus indicating qualitatively that the reductant competes very effectively with O_2 for transient **2** under these conditions. These spectral changes are also consistent with those seen in deaerated solutions of 1 with GSH.

A logical mechanism for trapping by GSH would be the one electron reduction of **2** to give the Cr(III) product *trans*-Cr(cyclam)$(OH)(ONO)^+$ (or/and the conjugate acid *trans*-Cr(cyclam)$(H_2O)(ONO)^{2+}$) (Scheme 1). The spectrum of the photoproduct formed after exhaustive photolysis in deaerated solution with added GSH (Figure 1A) displays ligand field (d−d) absorption bands at 332 and 538 nm with the approximate extinction coefficients 550 and 45 M^{-1} cm^{-1}, respectively. For comparison, the spectrum of **1** has bands at 336 and 476 nm (267 and 40 M^{-1} cm^{-1}) and that of the

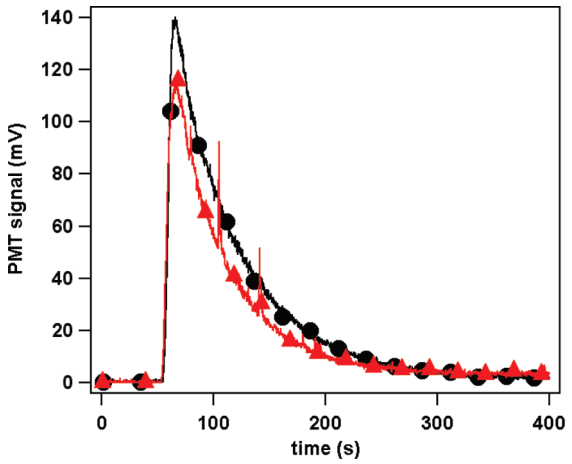


Figure 3. Photoinduced release of NO from **1** (150 μM) with continuous He purging after 5 s photolysis with 436 nm light. The medium is aqueous pH 7.4 phosphate buffer solution at 25 °C with (red triangles) and without (black circles) 5 mM GSH. The integrated area beneath each curve corresponds to the total amount of NO released.

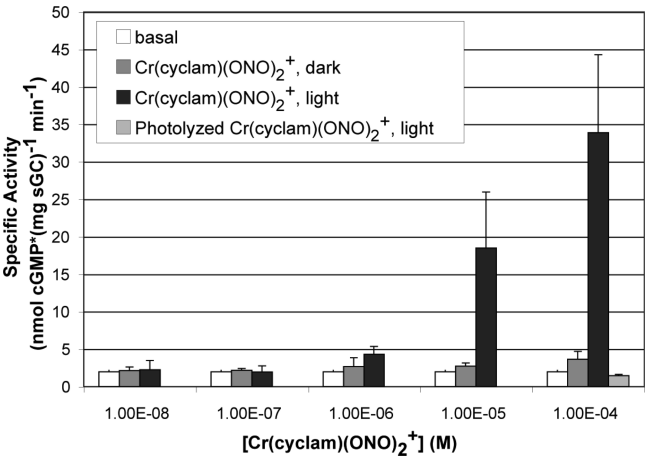


Figure 4. Graph of the specific activity (nmol cGMP produced per mg sGC per min) of sGC as measured by mol cGMP produced after incubation of 0.035 mg of the enzyme with *trans*-Cr(cyclam)-$(ONO)_2^+$ at 37 °C for 10 min. Activation over basal level, which did not contain added **1**, is significant for concentrations of with *trans*-Cr(cyclam)$(ONO)_2^+$ > 1 μM for the photolyzed samples. The activity of the sample where a prephotolyzed aliquot of **1** (100 μM) was added to the solution was the same as the control.

trans-Cr^{III}(cyclam)$(OH)_2^+$ complex displays bands at 340 and 519 nm (40 and 31 M^{-1} cm^{-1}).[42] Qualitatively the spectrum of the photoproduct is quite similar to both of these with the exception that the extinction coefficient of the higher energy band is significantly larger. One potential explanation would be that the proposed product of GSH trapping (Scheme 1) is not centrosymmetric, so the ligand field transitions are no longer strictly Laporte forbidden. However, regardless of the exact identity of the chromium(III) product, it is clear that the presence of GSH strongly enhances the net photoreactivity of deaerated solutions of **1** and that this behavior is relevant to the photochemistry expected under the hypoxic or anoxic environment of the tissues of solid tumors. The photolysis in solutions with GSH also prevents the formation of higher oxidation state chromium complexes, alleviating concern for the generation of these potentially toxic species.

In addition to formation of the Cr(III) photoproduct, 436 nm photolysis of **1** in the presence of GSH also leads to significant NO release. As shown in Figure 3, the amount of NO produced from samples with and without GSH is approximately the same in solutions of **1** continuously purged with helium. At first impression this result, obtained using the nitric oxide analyzer, seems inconsistent with the spectroscopic observations illustrated by Figure 1, where there was little apparent photoreaction in deaerated solutions when GSH was not present. The difference is the use of He to entrain NO from the solution during the NOA experiment. Under the latter conditions, the NO generated photochemically appears to be removed from the solution at a rate significantly faster than that of the back reaction with **2**.

Quantitative evaluation of the NO released as measured by the NOA, relative to the intensity of the light absorbed by the solutions, allowed calculation of the quantum yields for NO generation (Φ_{NO}). Under these conditions (continuous purging with flowing He), the values determined for Φ_{NO} were 0.25 for anoxic solutions containing added GSH (5 mM) and ~0.26 for those without added GSH. Notably, the quantum yield for the disappearance of **1** under 436 nm photolysis in aerated solutions was previously determined from changes in the absorption spectrum to be 0.27.[5,6,43] Within experimental uncertainties, this value is indistinguishable from the Φ_{NO} measured using the NOA. The similarity of the photochemical quantum yields measured under three markedly different conditions (aerated, deaerated, and with GSH) strongly suggests that the primary photoreaction is the same in each case, namely the β-cleavage of the CrO-NO bond indicated by eq 1 and Scheme 1. The different net outcomes are the result of the oxidative and reductive pathways that compete with the back reaction.

sGC Activation. A primary bioregulatory role of nitric oxide in mammalian physiology is its activation of soluble guanylyl cyclase, an enzyme that catalyzes the conversion of guanylyl triphosphate (GTP) to cyclic guanylyl monophosphate (cGMP).[14] sGC is the major cellular "receptor" for NO and mediates a wide range of physiological effects including vasodilatation and neurological function through elevation of intracellular cGMP levels. The high sensitivity of sGC toward NO is not only vital to NO's biological function (s) but also makes sGC activation a very sensitive test for NO release[15] from a thermal or photochemical precursor.

Figure 4 illustrates the result of incubating various solutions of **1** over the concentration range 10^{-8}–10^{-4} M with sGC using an enzyme-linked immunosorbent assay (ELISA) to evaluate the enzymatic production of cGMP. The basal specific activity of the sGC preparation under these conditions was 1.96 ± 0.22 (the units are nmol GMP per mg protein per min). Addition of *trans*-[Cr(cyclam)$(ONO)_2$]BF_4 solution had no significant effect on sGC activity up to a net concentration of 1 μM and led to only marginal activity increases up to 100 μM if the samples were kept in the dark at 37 °C. In contrast, irradiation of these systems with the polychromic light from a compact fluorescent lamp (10 min; spectrum in Supporting Information Figure S2), gave measurable effects on the sGC activity for preparations containing as little as 1 μM **1** and marked increases for solutions to which higher concentrations of **1** had been added. Control samples to which solutions of prephotolyzed *trans*-[Cr(cyclam)$(ONO)_2$]BF_4 (100 μM) were added showed no enhancement of sGC activity. Thus, we conclude that the photoinduced activity is due only to the NO released during

Table 1. Comparison of the Specific Activity of 0.035 mg Samples of sGC at 37 °C and pH 7.4 under Basal Conditions and after Activation by 10 min Incubation with Various Concentrations of *trans*-Cr(cyclam)$(ONO)_2^+$ (in the Dark or with Light Irradiation from a 14 W CFL) or DEA/NO[a]

		dark		irradiated	
activator	activator concentration, μM	specific activity[b]	activation[c]	specific activity[b]	activation[c]
none (sGC basal)		2.0 ± 0.2			
1	0.01	2.2 ± 0.4	1.1	2.2 ± 1.2	1.1
1	0.1	2.2 ± 0.2	1.1	2.0 ± 0.8	1.0
1	1	2.7 ± 1.2	1.4	4.3 ± 1.1	2.2
1	10	2.7 ± 0.4	1.4	18.5 ± 7.5	9.4
1	100	3.7 ± 1.0	1.9	34 ± 10	17
prephotolyzed **1**	100			2.9 ± 0.9	1.5
DEA/NO	1	26 ± 5	13		
DEA/NO	10	115 ± 7	58		
DEA/NO	100	133 ± 16	67		

[a] Using an ELISA assay. See text. [b] Units of activity are nmol cGMP/min/mg. [c] Activation = ratio of specific activity divided by basal activity.

the photolysis and not to the Cr photoproduct. The enhanced activity at higher concentrations of **1** can be attributed to higher release of NO as more light is absorbed by **1** because the light source was the same for all samples. The rate of NO production is equal to $\Phi_{NO} \times I_a$, where Φ_{NO} is the quantum yield for NO photogeneration from **1** and I_a is the intensity of light absorbed by **1**. Thus, at higher [**1**], more light is absorbed by the NO precursor and more NO is formed.

Table 1 compares the activation of sGC by photoinduced NO release from **1** to that by the thermal NO precursor, DEA/NO, which generates 2 equiv of NO per mol with a rate constant of 5.4×10^{-3} s^{-1} (in pH 7.4 phosphate buffer at 37 °C).[44] Under these conditions, complete release of NO from DEA/NO would occur within the 10 min incubation time. For comparable concentrations of **1**, the net activation upon photolysis is substantially smaller. This is not surprising, given that the photoreaction would be incomplete owing to the weak visible absorbances of **1** and the low-intensity light source utilized. Most importantly, the results in Figure 3 and in Table 1 clearly show that one can generate biologically relevant quantities of NO from the excitation of μM concentrations of **1** with low-intensity visible light.

Vasorelaxation. NO-mediated activation of sGC leads to conversion of GTP to the second messenger cGMP, and this process is ultimately responsible for vasorelaxation, thus having a major role in blood pressure regulation[3] and control of local blood flow. Arterial vasorelaxation has long been used to evaluate the therapeutic potential of NO donors, both photochemically and thermally activated.[35,38,44−50] In this context, we have investigated the impact of **1** on preconstricted porcine coronary arterial rings in order to confirm the efficacy of NO delivery from the visible range photolysis of *trans*-[Cr(cyclam)$(ONO)_2$]BF_4 solutions.

Figure 5A shows the concentration−response curve for solutions of **1** in the dark or exposed to light from a portable LED light source (centered at 470 nm, spectrum in Supporting Information, Figure S3) positioned at a fixed distance (10 cm) from the myography wells. Clearly when solutions of **1** bathing the arterial rings were irradiated with the 470 nm light, significant vasorelaxation was seen and this effect was dependent on the concentration of **1**, with a threshold of < 100 nM. No effect on the ring tension was noted for solutions of **1** up to 30 μM in the dark nor for analogous solutions exposed to ambient room light under these conditions. A third control experiment shown in Figure 4A illustrates the effect of analogous concentrations

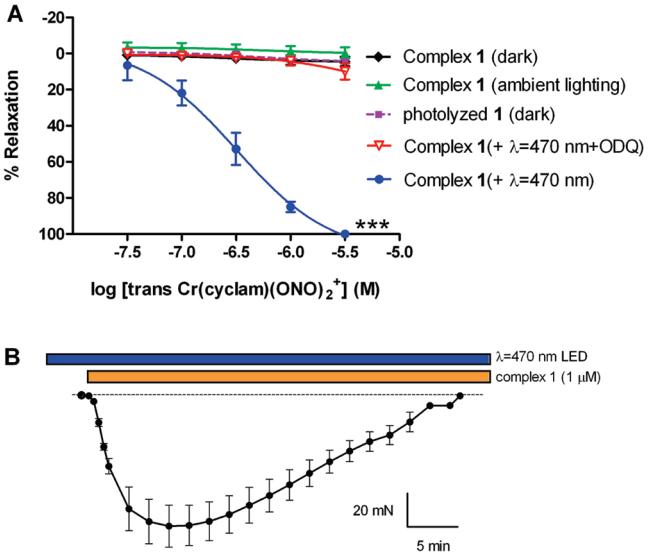


Figure 5. (A) Effect of **1** on vasorelaxation in endothelium-denuded porcine coronary arterial rings in the dark (black diamonds), ambient light (green triangles), or under exposure to LED light (470 nm; blue circles). The vasodilator effect of exposed **1** was abolished in the presence of the sGC inhibitor, ODQ (red open triangles) and was not seen with the photolyzed product of in the dark (**1** (purple squares; all mean ± SEM; $n = 5-9$; ***$P < 0.001$; 2-factor ANOVA 470 nm irradiated vs dark). (B) Time course of the effect of irradiated (470 nm LED, 10 cm) 1 μM (~EC_{80} concentration) complex **1** on arterial tone in denuded arterial rings. It is important to note that irradiation starts before **1** is added and that both irradiation and drug exposure are maintained until after full recovery has been achieved (mean ± SEM; $n = 4$).

of prephotolyzed **1** in arterial rings: the results confirmed that prephotolyzed 1 failed to cause any vasodilatation over this concentration range.

Thus, the experiments summarized by Figure 5A clearly show that the observed relaxation in arterial rings is attributable to the NO generated by photolysis of **1**, and not to the complex itself, nor to other photoproducts. Under these conditions, the log IC_{50} for solutions of *trans*-Cr(cyclam)-$(ONO)_2^+$ is -6.5 ± 0.2 (IC_{50} = ~300 nM), as assessed from a sigmoidal best-fit curve of the experimental results plotted in Figure 4 (Graphpad Prism software v5.0).

The response profile obtained with irradiation of a submaximal concentration of **1** is illustrated in Figure 4B. Photolysis of a solution of **1** at a concentration (1 μM)

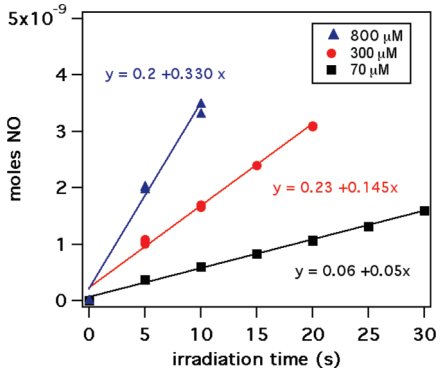


Figure 6. Calibration of the 470 nm LED light source at 10 cm irradiation distance. Solutions of three different concentrations of **1** were irradiated for set amount of times (5−20 s), and the moles of NO released during that time were measured by the NOA. The solutions were 15 mM phosphate buffered saline at pH 7.5 at ambient temperature. The linear fits for the NO flux per irradiation time are given by the slopes in the equations.

corresponding to ~IC_{80} caused a response that peaked in ~10 min and slowly recovered back to baseline within ~40 min. These results suggest that, within this period, **1** was completely photolyzed under the exposure conditions of this experiment and further emphasizes that the effect is due to a factor generated under photolysis that is transient in nature (i.e., not one of the stable products of photolysis).

To confirm that the relaxation of the arteries occurs through NO-activation of sGC, identical vasorelaxation experiments were carried out after incubation of the arteries with the sGC inhibitor ODQ. As shown in Figure 5A, ODQ pretreatment abolishes the vasodilator effect of **1** under irradiation.

Calibration of NO Release. The rate of photolysis-initiated NO release from solutions of the *trans*-[Cr(cyclam)-$(ONO)_2$]BF_4 salt under the influence of the 470 nm blue LED light source was determined using the NOA as described in the Experimental Section. Different concentrations of solutions of **1** in 15 mM phosphate buffer at pH 7.4 and 25 °C were irradiated while continuously flushing with helium for increasing time periods with the light source positioned at a distance of 10 cm in analogy to the distance used in the myography experiments described above. The amount of NO produced was measured with the NOA. As shown in Figure 6, linear fits were obtained for plots of the quantity of NO released (in moles) vs irradiation time for three separate concentrations of **1**. Thus, it is clear that under these conditions the NO release is dependent both on the concentration of the precursor and on the irradiation time. (Although not specifically demonstrated by these experiments, NO release would also be dependent on the intensity of the light source.) Dividing the slopes of these plots by the concentration of **1** gave the value ~550 ± 175 nmol of NO per molar concentration of **1** per second under this specific set of conditions. This gives a calibration of the system, which can be used to estimate the amount of NO generated from irradiation with the blue LED source. For the vasorelaxation experiments described above, the 3 min irradiation time with the blue LED of a 300 nM solution of **1** at a distance of 10 cm would correspond to ~0.03 nmol NO released in the 10 mL well, which would give a concentration of ~3 nM. This agrees with the low (nM) NO concentrations known to effect sGC activation and arterial vasorelaxation.[18]

Summary

These experiments confirm that the Cr(III) nitrito complex *trans*-Cr(cyclam)$(ONO)_2^+$ is an effective photochemical precursor for NO release in vitro even in a reducing, hypoxic environment of the type expected for solid tumors. Nitric oxide release from **1** was demonstrated using relatively low intensity, visible light sources. Moreover, very low concentrations (~100 nM) of this precursor proved to be sufficient to effect sGC activation and arterial ring vasorelaxation via photochemical NO release. No sGC activity or vasorelaxation were seen without light excitation of **1**. Biological activity at these precursor concentrations is quite remarkable given the very low visible range absorbances characteristic of the *trans*-Cr-(cyclam)$(ONO)_2^+$ cation. The efficacy of **1** in this regard can be attributed to the large quantum yield for NO generation (Φ_{NO} = ~0.26) in the visible region as well as to the high sensitivity of the biological assays. Ongoing studies are directed toward modifying **1** in order to introduce strongly light absorbing antennas with desirable optical properties and biological specificity to enhance the effective delivery of NO for potential therapeutic applications.[11,12,43,51] Parallel studies in other laboratories are following a similar strategy.[52−54] The resulting greater light adsorption should enhance the rate of NO generation.

Nonetheless, the present results demonstrate that despite the weak absorption bands at longer wavelengths, *trans*-Cr-(cyclam)$(ONO)_2^+$ is itself a very promising photochemical NO precursor. Sufficient NO is released with visible light irradiation of a low concentration solution ($< 1\ \mu M$) of **1** to lead to significant activation of sGC as well as of arterial relaxation. The use of a portable LED visible light source for this purpose is also promising for medical applications, as one can envision that such systems allow for irradiation without the necessity of more expensive, high intensity light sources.

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Supporting Information Available: Spectral changes when **1** is irradiated in aerated solution and the spectra of the CFL and LED light sources. This material is available free of charge via the Internet at http://pubs.acs.org.

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