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A "Dual-Function" Photocage Releasing Nitric Oxide and an Anthrylmethyl Cation with a Single Wavelength Light

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The design and development of new anticancer agents is one of the most challenging enterprises of the modern scientific research. Cancer chemotherapy mainly relies on the use of drugs which interact with DNA, inducing modification via different mechanisms including electron transfer, hydrogen-atom abstraction and generation of diffusible intermediates.[1]

In recent years, nitric oxide (NO) has come to the limelight not only to be a pleiotropic bioregulator of key physiological processes in living bodies,[2] but also for its promising anticancer activity.[3] In this regard, DNA has been labeled as the main target of NO, which can induce deamination of the nucleobases producing abasic sites and, eventually, leading to strand breaks.[4] Besides, recent developments in drug design witness an upsurge of interest in DNA-alkylating agents as potential anticancer compounds.[5] In these cases, the antineoplastic activity is mainly the result of the reaction between carbocation intermediates with the 2-amino group of guanine residues in DNA.[6] An important point to be outlined is that both NO and carbocation species can act as a double-edge sword either inhibiting or encouraging the tumor proliferation depending on the dose.[7] Therefore, rapidity of delivery and precise spatiotemporal control of these transient species are fundamental criteria that would be meet in view of therapeutic applications. Using light as external trigger provides the great opportunity to satisfy both the above requisites in the case of "caged molecules",[8] with the additional advantage of not affecting important physiological parameters such as pH, temperature and ionic strength. In fact, the fast response of the photochemical reactions associated to their instantaneous initiation/stopping depending on the presence or not of the illumination, represent a powerful tool for the rapid introduction of bioactive species in the desired bio-environment with accurate control in space and time.

On these grounds, the development of compounds able to combine generation of NO with carbocation intermediates under the control of light stimuli can be a significant step forward in the development of smart "dual-function" anticancer drugs. Such compounds aim to exploit either additive or synergistic effects arising from the generation of two anticancer species in the same region of space with the final goal to maximize the efficiency of the treatment and, in principle, might be activated in vivo by laser sources coupled with fiber optics.

Motivated by our ongoing interest in developing photoactivable NO-releasing systems,[9] we report herein an intriguing example of a dual function photocage able to associate the generation of an anthrylmethyl carbocation to the release of NO, under the control of a low energy single wavelength light. The design of this photocage was inspired by an excellent work of Wang and co-workers[10] on a series of substituted derivatives of cupferron (**CP**), a spontaneous NO donor at room temperature[11] (Scheme 1a).[12] They showed that *O*-alkylation of **CP** leads to thermostable *O*-alkyl derivatives, which release NO only under light irradiation (Scheme 1b). However, since all these derivatives exhibit absorption in the extreme UV region, their practical use is strongly limited by the very high excitation energy ($\lambda_{exc}=254$ nm) required for the photolysis, which is not suited for bio-applications because absorbed by the biological environment. Furthermore, no mechanistic insights concerning the photoactivated NO release were provided.

To accomplish our goals we sought to harness the *O*-alkylation of **CP** by designing and synthesizing compound **1** (Scheme 1c).

Due to the presence of the anthracene chromogenic center, **1** offers the possibility to shift the excitation energy well above 300 nm, indispensable prerequisite in the context of bio-applications. We demonstrate that excitation of **1** with 390 nm light induces heterolytic cleavage of C–O bond leading to the formation of the 9-anthrylmethyl carbocation,

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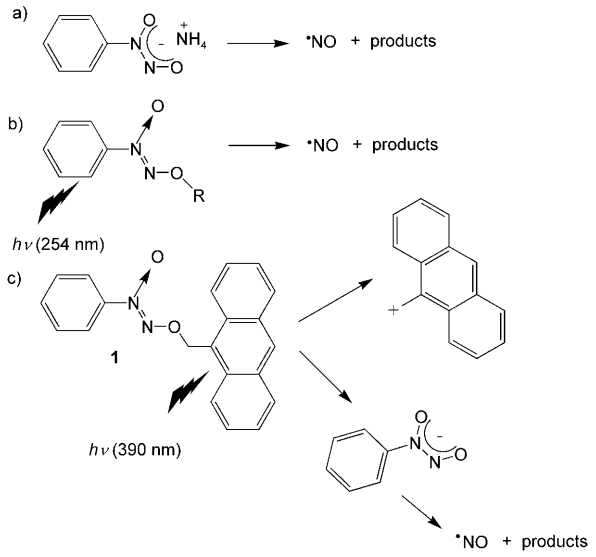


Scheme 1.

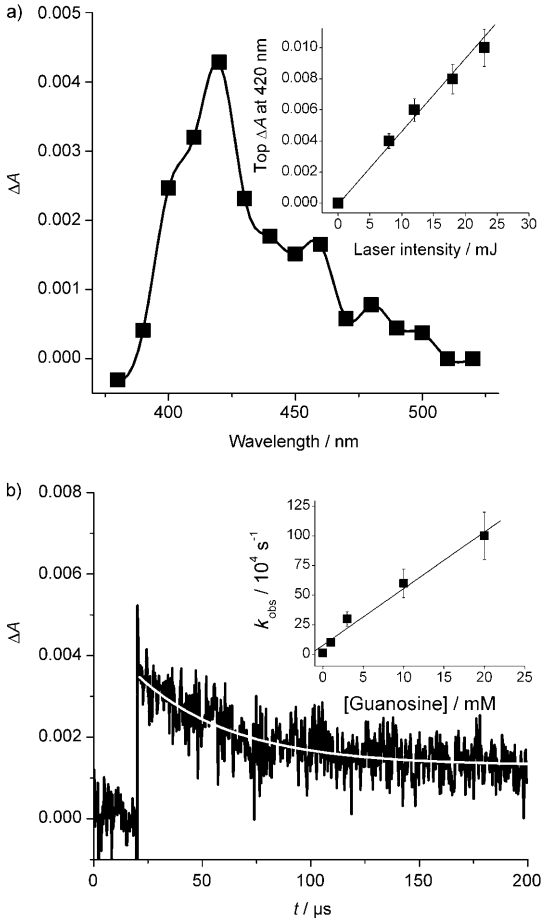


Figure 1. a) Transient absorption spectrum recorded 0.02 μs after 355 nm laser excitation of a phosphate buffer (10 mM, pH 7.4)/MeOH (1:1 *v*/*v*) solution of **1** (60 μM). The inset shows the top ΔA at 420 nm nm as a function of the laser intensity. b) Decay profile observed at 420 nm and related first-order fitting. The inset shows the plot of k_{obs} as a function of the concentration of guanosine.

able to react with nucleosides, and to the release of NO as a consequence of the **CP** decaging (Scheme 1c).

Compound **1** was easily synthesized in one step. Its irradiation with low energy light excitation in a mixture phosphate buffer (10 mM, pH 7.4)/CH_3OH (1:1 *v*/*v*) leads to a satisfactory photodegradation with a quantum yield $\Phi \cong 0.05$. Laser flash photolysis is a powerful tool to directly demonstrate the involvement of the anthrylmethyl carbocation as the key intermediate in the photodecomposition. Figure 1a shows the spectrum observed upon 355 nm laser excitation of a solution of **1**. This transient is generated via a monophotonic mechanism (see inset Figure 1a) and exhibits a maximum at 420 nm, a shoulder at shorter wavelengths and an unstructured weaker absorption extending up to about 500 nm. Its decay was first-order with rate constant $k_0 \cong 1 \times 10^4\ s^{-1}$ (Figure 1b), unaffected by oxygen, and accelerated by azide, an excellent scavenger of cations. According to extensive literature data on arylmethyl carbocation derivatives,[13] these spectral and kinetic characteristics suggest the assignment of this transient spectrum to the 9-anthrylmethyl carbocation. It is worthy to note that the cation is completely formed within the 6 ns laser pulse, which means that its formation rate is $> 1.6 \times 10^8\ s^{-1}$. The potential of this transient species to react with the guanine moiety was tested by quenching experiments carried out in the presence of increasing amounts of guanosine. The slope of the straight line of the plot $k_{obs} = k_0 + k_q$[guanosine], afforded a bimolecular quenching constant $k_q \cong 4 \times 10^7\ M^{-1} s^{-1}$ (inset Figure 1b), in good agreement with values reported for similar carbocations with amine derivatives.[13e]

Photogeneration of the anthrylmethyl carbocation implies the consequent release of the **CP** counterpart, which is expected to rapidly generate NO at room temperature. The most convenient methodology to unambiguously demonstrate the NO generation is the direct and in real-time monitoring of this radical species. To this end, we used an ultrasensitive NO electrode, which directly detects NO concentration by an amperometric technique. The results illustrated in Figure 2 provide clear evidence that compound **1** is stable to the release of NO in the dark. In contrast, light irradiation induces constant generation of NO after a brief initiation period, in according to the initial photodecaging of **CP** which is the actual precursor of NO.

To get more insights into the mechanism responsible for the photodecomposition of **1** we combined steady-state and time-resolved experiments by using suitable model compounds. The absorption spectrum of **1** in the 300–450 nm does not show any significant difference with respect to that of 9-anthracene methanol, chosen as a model compound, suggesting negligible mutual interactions between the anthracene and the **CP** moieties in the ground state (Figure 3a). In contrast, the fluorescence quantum yield of **1** is more than two orders of magnitude smaller than that of the model compound (Figure 3b).

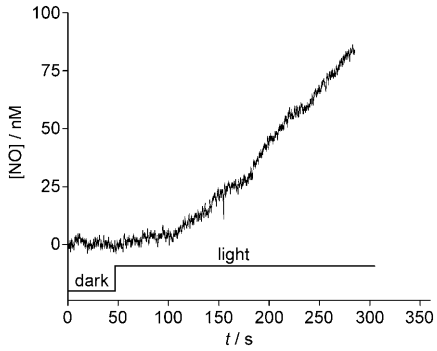


Figure 2. NO release observed upon irradiation of a N_2-saturated phosphate buffer (10 mM, pH 7.4)/MeOH (1:1 *v*/*v*) solution of **1** (60 μM) with 390 nm light excitation.

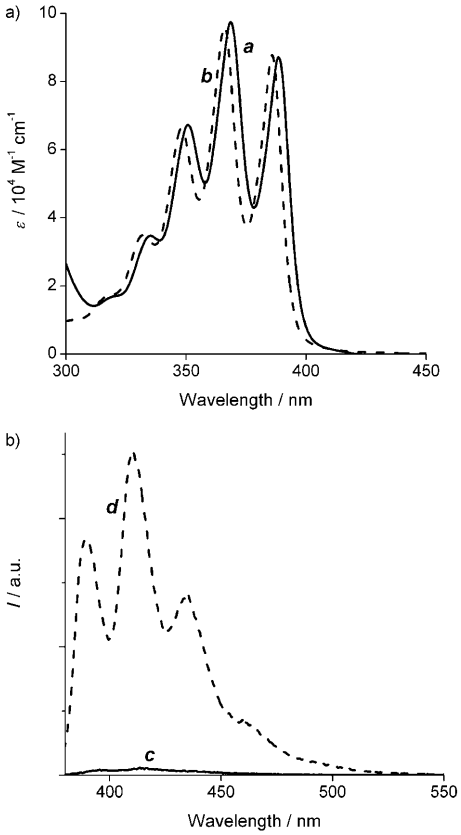
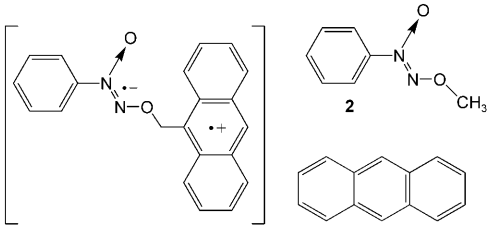


Figure 3. a) Absorption and b) fluorescence emission spectra (λ_{exc} = 365 nm) of **1** (***a***,***c***) and 9-anthracene methanol (***b***,***d***) in phosphate buffer (10 mM, pH 7.4)/MeOH (1:1 *v*/*v*).

Since the lowest excited singlet state of the anthracene chromophore (that is selectively excited) lies about 0.8 eV below that of the **CP** unit,[14] a photoinduced energy transfer between these two units is clearly endoergonic and thus, cannot be responsible for the drastic fluorescence quenching observed. This suggests the involvement of a non-emissive intramolecular exciplex with some charge-transfer (CT) character as a precursor for the heterolytic C–O bond cleavage (Scheme 2) according to what observed for other anthracene derivatives.[15] This proposal is supported by the large change in free energy calculated for this process, $\Delta G_{CT} \cong -1.1$ eV[16], which is strongly corroborated by the intermolecular quenching experiments carried out by using anthracene and the *O*-methyl derivative of **CP** (**2**) as model compounds (Scheme 2).



Scheme 2. Schematic illustration of the singlet-exciplex intermediate derived from **1** (left) and the model compounds used for the intermolecular quenching experiments (right).

As shown in Figure 4a, the fluorescence of anthracene is effectively quenched by **2** according to the Stern–Volmer equation, $I_0/I = 1 + k_q\tau[\mathbf{2}]$ where I_0 and I are the fluorescence intensities of anthracene in the absence and in the presence of **2**, respectively, k_q is the bimolecular quenching constant and τ is the singlet lifetime of the anthracene. From the slope of the linear plot and the value of τ (ca. 4 ns), a value for $k_q \cong 6 \times 10^9\,\text{M}^{-1}\text{s}^{-1}$ was determined at room temperature. Laser flash photolysis experiments provide a clear proof that the quenching process occurs through an electron transfer mechanism. In fact, as shown in Figure 4b, 355 nm laser excitation of a solution of anthracene in the presence of **2** 0.06 M (>99% quenching) originates the typical transient absorption of the anthracene radical cation characterized by its diagnostic absorption with maxima at 710 and 420 nm.[17]

In summary, we have designed and synthesized a novel dual function photocage that, to the best of our knowledge, represents the first example of molecular system able to release NO and a carbocation species upon absorption of a single wavelength light. We have demonstrated that the photodecaging process is most likely triggered by a fast intramolecular charge transfer and that the anthrylmethyl carbocation generated is able to react with guanosine, an essential requisite in view of its anticancer properties. This photocage satisfies a number of key criteria for potential biological applications including stability in the dark, satisfactory photochemistry, adequate absorption at wavelengths longer than 300 nm and a high decaging rate constant. Finally, it is important to point out that since the antineoplastic activity of both NO and carbocations comes from the direct reaction of these species with DNA (see above), the presence a well-known DNA intercalator such as the anthracene moiety into the structure of the photocage offers, in principle, the additional advantage to generate these anticancer species in the proximity of their main target. Study addressed to shed light into the binding affinity of **1** with DNA and to evaluate the

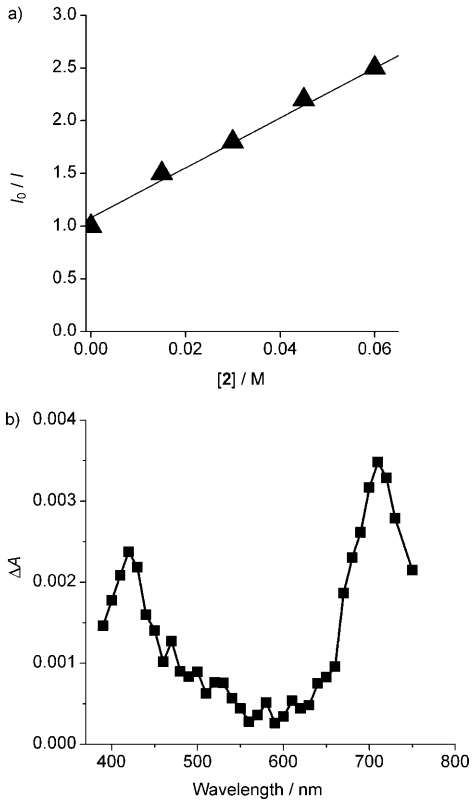


Figure 4. a) Stern–Volmer plot for the fluorescence quenching of anthracene by **2** at room temperature. b) Transient absorption spectrum recorded 0.05 μs after 355 nm laser excitation of a phosphate buffer (10 mM, pH 7.4)/MeOH (1:1 *v*/*v*) solution of anthracene in the presence of **2** 0.06 M.

extent of the photoinduced DNA photocleavage are currently underway in our laboratory.

Experimental Section

Syntheses: Compounds **1** and **2** were synthesized in one step according to the procedure reported in ref. [10]. Briefly, 9-chloromethyl anthracene (in the case of **1**) or methyl iodide (in the case of **2**) were injected through a septum to a DMF solution of **CP** cooled at 0 °C and stirred overnight. The reaction mixtures were diluted with water, extracted with CH_2Cl_2 and, after solvent evaporation, the products were purified by silica gel chromatography using cyclohexane/ethyl acetate 80:20.

(*Z*)-2-(9-Anthrylmethoxy)-1-phenyldiazene 1-oxide (1): Elemental analysis calcd (%) for $C_{21}H_{16}N_2O_2$: C 76.81, H 4.91, N 8.53; found: C 76.05, H 4.82, N 8.21; ESI-MS: *m*/*z* (%): 351.1 (100) $[M+Na]^+$; ^{1}H NMR ($CDCl_3$, 500 MHz): $\delta = 8.55$ (s, 1H), 8.50 (d, 2H, $J = 8.5$ Hz), 8.04 (d, 2H, $J = 8$ Hz), 7.86 (dd, 2H, $J_1 = 8.4$, $J_2 = 7.5$ Hz), 7.61 (dd, 2H, $J_1 = 8$, $J_2 = 7.4$ Hz), 7.50 (m, 2H), 7.45 (m, 1H), 7.40 (m, 2H), 6.45 ppm (s, 2H).

(*Z*)-2-Methoxy-1-phenyldiazene 1-oxide (2): Elemental analysis calcd (%) for $C_7H_8N_2O_2$: C 55.26, H 5.30, N 18.41; found: C 56.12, H 5.54, N 19.03; ESI-MS: *m*/*z* (%): 175.1 (100) $[M+Na]^+$; ^{1}H NMR ($CDCl_3$, 500 MHz): $\delta = 7.80$ (d, 2H, $J = 7.8$ Hz), 7.67–7.55 (m, 3H), 4.00 ppm (s, 3H).

Steady-state photolysis: Irradiation was performed in a thermostated quartz cell (1 cm pathlength, 3 mL capacity) by using the monochromatic radiation (390 nm) of a fluorimeter Fluorolog-2 (mod. F-111) as light source. Photodecomposition of **1** was followed by high-performance liquid chromatography (HPLC) by using a LiChroCart RP-18 column (5 μm packing, 4×250 mm; Hewlett–Packard) and eluting with CH_3CN/water (3:2 *v*/*v*). The quantum yield for the photodecomposition of **1** was determined by using the following equation:

$$\Phi = -\Delta[\mathbf{1}]V/\Delta t I_0 F$$

where $\Delta[\mathbf{1}]/\Delta t$ is the rate of disappearance of **1**, V is the volume of the irradiated solution, I_0 is the intensity of the incident photons and F is the fraction of the photons absorbed by **1** at the excitation wavelength.

Laser flash photolysis: The samples were excited with the third harmonic of a Nd-YAG Continuum Surelite II-10 laser system (pulse width 6 ns FWHM, at $\lambda = 355$ nm) and the excited solutions were analyzed at a right angle geometry using a mini mLFP-111 apparatus developed by Luzchem Research. Briefly, the monitoring beam was supplied by a ceramic xenon lamp and delivered through quartz fiber optical cables. The laser pulse was probed by fiber that synchronized the mLFP system with a Tektronix TDS 3032 digitizer operating in the pre-trigger mode. The signals from a compact Hamamatsu photomultiplier were initially captured by the digitizer and then transferred to a personal computer that controlled the experiment with Luzchem software developed in the LabView 5.1 environment from National Instruments. The energy of the laser pulse was measured at each laser shot by a SPHD25 Scientech pyroelectric energy monitor. Oxygen was removed by vigorously bubbling the solutions with a constant flux of argon previously passed through a water trap. The solution (in a flow cell of 1 cm pathlength) was renewed after each laser shot. The sample temperature was 295±2 K.

NO detection: NO release was measured with a World Precision Instrument, ISO-NO meter, equipped with a data acquisition system, and based on direct amperometric detection of NO with short response time (<5 s) and sensitivity range 1 nM–20 μM. The analog signal was digitalized with a four-channel recording system and transferred to a PC. The sensor was accurately calibrated by mixing standard solutions of $NaNO_2$ with 0.1 M H_2SO_4 and 0.1 M KI according to the reaction:

$$4\,H^+ + 2\,I^- + NO_2^- \rightarrow 2\,H_2O + 2\,NO + I_2$$

NO measurements were carried out with the electrode positioned outside the light path in order to avoid false NO signal due to photoelectric interference on the ISO-NO electrode.

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Keywords: antitumor agents · DNA · light · nitric oxide · photochemistry

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