High-Performance Caged Nitric Oxide: A New Molecular Design, Synthesis, and Photochemical Reaction

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Endogenous nitric oxide is an important messenger molecule in both the brain[1] and blood vessels[2] and acts as a cytotoxic agent in the immune system.[3] Since Kaplan reported the photolytic release of adenosine 5′-triphosphate (ATP) from a protected derivative that has no bioactivity, i.e., ATP *o*-nitrobenzyl ester,[4] these photosensitive precursors, called caged compounds, have become an important tool in the biosciences, especially in exploring signal transductions in living systems.[1b,5,6]

The great majority of known caged compounds are *o*-nitrobenzyl esters of desired bioactive molecules.[5] Soon after we started this work, Makings and Tsien reported *o*-nitrobenzyl ester caged nitric oxides (NOs).[7] The quantum yields of NO formation (Φ_{NO}) for their photochemical uncaging reaction were very poor (0.02–0.05) and NO chemical yields were only 30–54%.[7] The efficiency of $K_2Ru(NO)Cl_5$, used as a caged NO by Murphy et al., is also very low.[6] Here, we report an extremely high-performance caged NO having a new molecular design.

The following three factors were considered in our molecular design of caged NO: (a) Because NO is a free radical, two NO molecules must be produced from a single caged NO molecule so that the remaining cage counterpart is a nonradical species after the photochemical reaction to produce the NO (uncaging). (b) Uncaging proceeds in a high Φ_{NO}. To achieve high Φ_{NO}, the weaker the bond between the NO and the cage counterpart in the caged NO, the better. If the bond is very weak, however, thermal uncaging occurs even at room temperature.[8] (c) The caged NO requires a large molar extinction coefficient.

Fulfilling the above requirements, we designed *N*,*N*′-bis-(carboxymethyl)-*N*,*N*′-dinitroso-*p*-phenylenediamine ($\mathbf{I_A}$) as a water-soluble caged NO and *N*,*N*′-dimethyl-*N*,*N*′-dinitroso-*p*-phenylenediamine ($\mathbf{I_B}$) as a lipid-soluble caged NO.[9] The homolysis of one N–N bond may induce homolysis of the second N–N bond, producing the second NO either stepwise or concerted (Scheme 1). The driving force of the expected reaction is the formation of the two stable free radicals and
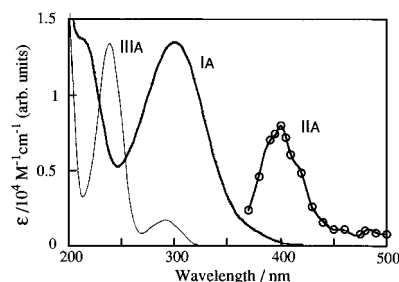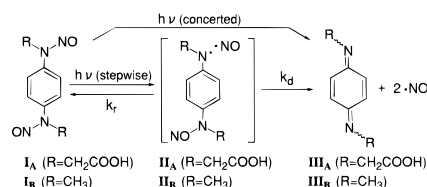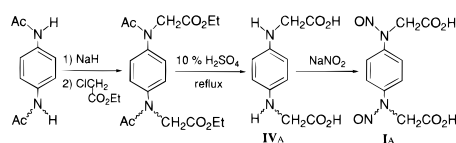

Figure 1. Electronic spectra. $\mathbf{I_A}$: in 0.1 M sodium phosphate buffer, pH 7.4. $\mathbf{II_A}$: Transient spectrum obtained by LFP of $\mathbf{I_A}$ in 0.1 M sodium phosphate buffer, pH 7.4. $\mathbf{III_A}$: in 0.1% aqueous phosphoric acid.

Scheme 1

Scheme 2

formation of new C–N π-bondings in the broken-cage residue (**III**). Although homolytic cleavage of the N–N bond in mono-*N*-nitroso compounds occurs under irradiation, the radical pairs recombine, providing a starting material or a *C*-nitroso compound.[10,11]

$\mathbf{I_A}$ was synthesized by the pathways shown in Scheme 2.[12a] $\mathbf{I_B}$ was prepared by the nitrosation of *N*,*N*′-dimethyl-*p*-phenylenediamine ($\mathbf{IV_B}$) with nitrous acid.[12b] Caged NOs have strong absorption bands in the UV region ($\mathbf{I_A}$ λ_{max} = 300 nm (ϵ = 13.5 mM^{-1} cm^{-1}) in a phosphate buffer of pH 7.4 (Figure 1); $\mathbf{I_B}$ λ_{max} = 299 nm (ϵ = 14.05 mM^{-1} cm^{-1}) in methanol). Without light, a solution of $\mathbf{I_A}$ in a buffer of pH 7.4 at 37 °C initiated no reaction.

HPLC analysis of the reaction mixture after partial photolysis yielded only one peak besides the peak of the remaining starting $\mathbf{I_A}$ under different conditions. The broken cage residue of $\mathbf{I_A}$ was identified as $\mathbf{III_A}$ by comparison with the retention time and electronic spectrum (λ_{max} 290 nm) of the authentic sample prepared by the oxidation of amino acid $\mathbf{IV_A}$ with potassium ferricyanate (Scheme 3).[13]

Inter- and intracellar NO-mediated signal transductions are initiated by the coordination of NO with guanylate cyclase, a heme enzyme.[14] To mimic this, $\mathbf{I_A}$ was photolyzed in a phosphate buffer solution of pH 7.4 containing mesotetrakis-(*p*-sulfonatophenyl)porphinatocobalt (TPPSCo), a heme model,

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(8) Thionitrite is a good example. Thionitrite photolysis readily produces NO;[8a,b] S–N bond homolysis, however, occurs at room temperature.[8c,d] (a) Barrett, J.; Debenham, D. F.; Glauser, J. *Chem. Commun.* **1955**, 248–249. (b) Barrett, J.; Fitzgibbons, L. J.; Glauser, J.; Still, R. H.; Young, P. N. W. *Nature* **1966**, *211*, 848. (c) Arnelle, D. R.; Stamler, J. S. *Arch. Biochem. Biophys.* **1995**, *318*, 279–285. (d) Trace heavy metal ions catalyze thionitrite decomposition to generate NO: Askew, S. C.; Barnett, D. J.; McAninly, J.; Williams, D. L. H. *J. Chem. Soc., Perkin Trans 2* **1995**, 742–745.
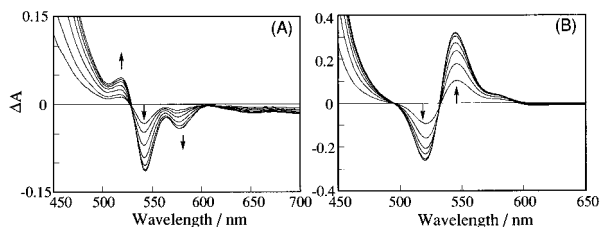
(9) In the distribution equilibrium experiments between benzene and 0.1 M sodium phosphate buffer solution at pH 7.4, distribution ratios for $\mathbf{I_A}$ ($K_{\mathbf{I_A}}$) and $\mathbf{I_B}$ ($K_{\mathbf{I_B}}$) were found to be $K_{\mathbf{I_A}} < 10^{-3}$ and $K_{\mathbf{I_B}} > 5 \times 10^2$, where K_I = [I]benzene/[I]water.

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(11) The photolysis of *N*-nitroso-*N*-methylaniline, for example, initiates photochromism of the compound through recombination of the intermediary radical pair: Hoshino, M.; Kokubun, H.; Koizumi, M. *Bull. Chem. Soc. Jpn.* **1970**, *43*, 2796–2800.

(12) (a) For $\mathbf{I_A}$: mp 145–6 °C (dec). Anal. Calcd for $C_{10}H_{10}N_4O_6$: C, 42.55; H, 3.55; N, 19.86. Found: C, 42.49; H, 3.62; N, 19.55. (b) For $\mathbf{I_B}$: mp 120–1 °C. Anal. Calcd for $C_8H_{10}N_4O_2$: C, 49.57; H, 5.18; N, 28.83. Found: C, 49.29; H, 5.20; N, 28.63.

Figure 2. Difference spectra from photolysis of I in the presence of porphinatocobalt. (A) Photolysis of I_A: $[I_A]_0 = 1.11$ mM, $[TPPSCo]_0 = 73.8$ μM in 0.1 M phosphate buffer of pH 7.4; $t = 0, 2, 4, 6, 8, 10$, and 12 min. (B) Photolysis of I_B: $[I_B]_0 = 0.78$ mM, $[TPPCo]_0 = 70$ μM in THF; $t = 0, 0.5, 1, 1.5, 3, 4$, and 5 min.
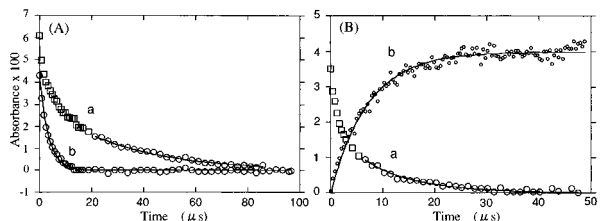

Figure 3. (A) Time courses for transient absorption at 405 nm in LFP of 69.6 μM I_A in pH 7.4 sodium phosphate buffer solutions. (a) Under Ar. (b) Under NO. (B) LFP of I_B in benzene under Ar. (a) Time course for transient absorption at 405 nm, $[I_B]_0 = 63.6$ μM. (b) Time course for raising absorption of TPPCo(NO) at 546 nm in the presence of 55 μM TPPCo, $[I_B]_0 = 14.7$ μM. All solid lines are first-order kinetics simulation curves.

Scheme 3
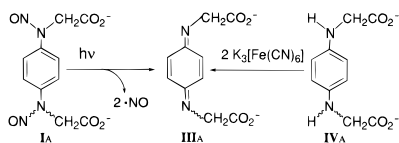
in an evacuated, sealed UV cuvette. I_B was uncaged in benzene containing mesotetraphenylporphinatocobalt (TPPCo). The spectrum change in photolysis (Figure 2) shows difference spectra obtained by subtracting the initial spectrum from those recorded at different photolytic uncaging time intervals. Difference spectra are identical to those obtained using authentic TPPSCo(NO) and TPPCo(NO) prepared by reacting TPPSCo and TPPCo with commercially obtained NO gas (λ_{max} 542 nm, λ_{min} 519 nm for TPPSCo(NO) and λ_{max} 545 nm, λ_{min} 520 nm[15] for TPPCo(NO)). The photolytic reactions of caged NOs are clean (Figure 2).

When solutions of I_A in pH 7.4 sodium phosphate buffer and I_B in benzene were subjected to laser flash photolysis (LFP) with 308 nm wavelength and a 20 ns laser pulse at 23 °C, transient species having visible absorption band (Figure 1) appeared immediately. The time courses of the decreasing absorbance at 405 nm consist of fast phases ($\square$, Figure 3, trace a) and slow phases ($\bigcirc$, Figure 3, trace a). Slow phases obeyed the first-order kinetic equation well ($-$, Figure 3) to yield $k_d = 2.96 \times 10^4$ s^{-1} for I_A in water and $k_d = 2.15 \times 10^5$ s^{-1} for I_B in benzene. Molecular oxygen did not alter the lifetime of the transient species, but NO accelerated decay. In a NO-saturated aqueous solution, the slow phase disappeared and the entire decay curve fitted a first-order kinetic equation with $k_{obsd} = 2.69 \times 10^5$ s^{-1} (Figure 3A, trace b). These observations and the NO trapping experiments, below, with TPPCo in benzene

reveal that the photolysis of I occurs stepwise via radical intermediate II, which disappears via elimination of the second NO and recombination to regenerate I.

According to this mechanism, II disappears via the kinetic equation $-d[II]/dt = (k_r[NO] + k_d)[II]$. In the presence of a large excess NO, the bimolecular reaction predominates over the unimolecular NO extrusion reaction of II to yield $k_{obsd} = k_r[NO]$. From the solubility of NO at 23 °C (1.95 mM)[16] and the k_{obsd} value, the second-order rate constant (k_r) for recombination of II_A and NO is calculated to be 1.38×10^8 M^{-1} s^{-1}. Since concentrations of caged NOs are 10^{-8}–10^{-6} M in biological applications, the bimolecular recombination of NO and II should be negligible in practical use.

When the LFP of I_B benzene solutions having a constant initial concentration in the presence of a large excess TPPCo was performed, time courses of rising absorption at 546 nm due to the formation of TPPCo(NO) obeyed the first-order kinetic equation (Figure 3B, trace b). Pseudo-first-order rate constants correlated linearly with [TPPCo] to obtain the second-order rate constant for the binding of NO and TPPCo (2.80×10^9 M^{-1} s^{-1}), which agrees with previously reported 7×10^8 M^{-1} s^{-1} in 2-methyltetrahydrofuran.[15] When the benzophenone T$-$T band absorbance ($\epsilon = 10\,300$) at 532.5 nm was used as a standard,[17] the quantum yield for the formation of TPPCo(NO), $\Phi_{TPPCo(NO)}$, was determined to be 1.97 ± 0.33 from LFP with $[I_B] = 7.2$ μM and [TPPCo] $= 60$ μM. In low TPPCo concentration, not all NOs could be captured by TPPCo. As was predicted in our molecular design, a single photon generates almost two NO molecules from these caged NOs.

To further substantiate uncaging efficiency, a solution of I_A (21.3 μM) in a 0.1 M sodium phosphate buffer solution at pH 7.4 was irradiated until all I_A was photolyzed under Ar in the presence of 0.23 mM 2-(4-carboxyphenyl)-3,3,4,4-tetramethylimidazoline-1-oxyl 3-oxide (carboxy-PTIO), which reacts with the NO fading blue (λ_{max} 561 nm, $\epsilon = 970$ M^{-1} cm^{-1}).[18] Light having a 300 ± 0.5 nm wavelength was provided from a 500 W xenon lamp through a monochromater. One mole of I_A was found to consume 1.98 ± 0.01 mol of carboxy-PTIO. Similarly, in irradiation of a tetrahydrofuran solution containing I_B and large excess amounts of 2-phenyl-3,3,4,4-tetramethylimidazole-1-oxyl 3-oxide (PTIO), 1 mol of I_B reacted with 2.05 ± 0.10 mol of PTIO. These observations reveal that 1 mol of caged NOs quantitatively yields 2 mol of NO upon uncaging.

The values of the quantum yield consuming PTIO, Φ_{PTIO}, were determined using a potassium ferrioxalate actinometer.[19] Values of the experimentally observed Φ_{PTIO} in uncaging were 1.87 ± 0.08 with 300 nm light, 1.6 ± 0.1 with 350 nm light for I_A, and 2.02 ± 0.03 for I_B with 300 nm light.[20] The quality of the caged compound is expressed by $\epsilon\Phi$. The $\epsilon\Phi$ values of I_A were 2.7×10^4 M^{-1} cm^{-1} with 300 nm light and 6×10^3 M^{-1} cm^{-1} with 350 nm light. This is much greater than those for reported *o*-nitrobenzyl ester caged NOs ($\epsilon\Phi = 15$–70 M^{-1} cm^{-1}).[7]

The assay of caged NOs on rat aorta vasorelaxation is currently underway at this laboratory. Preliminary results reveal that caged NOs are very high-performance and have no measurable toxicity.

(13) UV spectrum for III_A (Figure 1) was recorded by a multichannel detector for HPLC at the height of HPLC peaks. Quinoimines are unstable, so III_A was consecutively converted to secondary products. Since we were not interested in the reactions of III_A, we did not further examine secondary products.

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