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Fluorescein Analogue Xanthene-9-Carboxylic Acid: A Transition-Metal-Free CO Releasing Molecule Activated by Green Light

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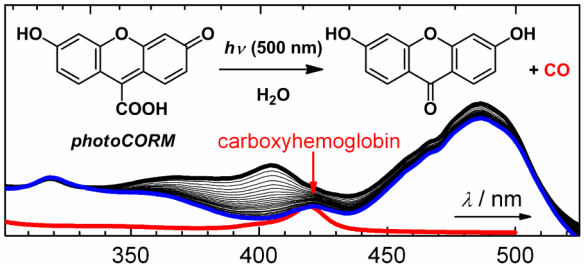
ABSTRACT



6-Hydroxy-3-oxo-3*H*-xanthene-9-carboxylic acid is introduced as the first transition-metal-free carbon monoxide releasing molecule activated by visible light (photoCORM). This water-soluble fluorescein analogue releases carbon monoxide in both water and methanol upon irradiation at 500 nm. When selectively irradiated in the presence of hemoglobin (Hb) under physiological conditions, released CO is quantitatively trapped to form carboxyhemoglobin (COHb). The reaction progress can be accurately monitored by characteristic absorption and emission properties of the reactants and products.

Carbon monoxide, one of the byproducts of the enzymatic heme catabolism by heme oxygenase, has been recognized as an essential physiological signaling molecule.[1] CO acts as an agent for tissue protection via its anti-inflammatory, antiproliferative, and antiapoptotic effects at cellular concentrations ranging from 10 to 250 ppm.

Various metal-based carbon monoxide releasing molecules (CORMs) that can be used to elicit various biological activities and for therapeutic applications have been introduced in the past decade.[2] Low toxicity, water solubility, and stability prior to the application are the most desirable properties of CORMs. Contrary to various small organic molecules, such as cyclopropenones,[3] 1,3-cyclobutanediones,[4] or 1,2-dioxolane-3,5-diones,[5] which liberate CO upon biologically adverse UV or near-UV (below 420 mn) irradiation, some transition-metal containing photoactivatable[6] CORMs (photoCORMs) that can be triggered by visible light[7] have been introduced recently. Mn-based photoCORMs, for example, polypyridyl metallodendrimers[8] and complexes

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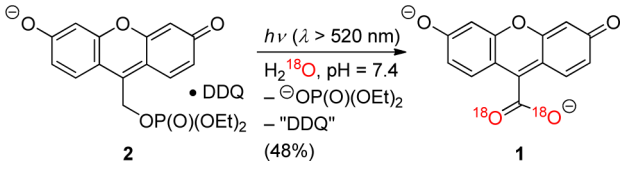
of various azaheteroaromatic ligands,[9] can release CO upon irradiation at 410 and > 500 nm, respectively.

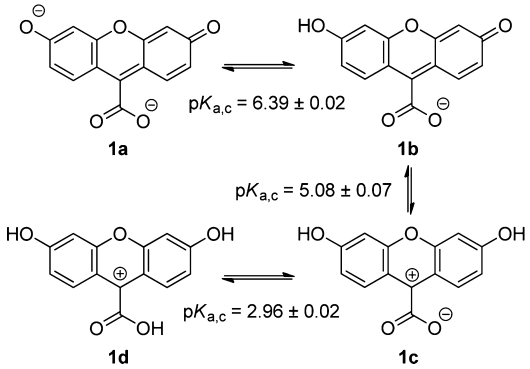
In this work, we introduce the first water-soluble, transition-metal-free CORM that can be activated by visible light. Released CO is shown to be quantitatively trapped by hemoglobin (Hb) under physiological conditions.

Synthesis and Physico-Chemical Properties of 1. 6-Hydroxy-3-oxo-3*H*-xanthene-9-carboxylic acid (**1**) is a fluorescein analogue possessing a nonaromatic substituent attached to the C9-position. The synthesis of this compound has been reported long ago.[10] However, following these procedures we obtained complex mixtures that did not contain any substantial amount of **1**. We also attempted to prepare this compound by several alternative synthetic pathways which were, unfortunately, unsuccessful (Scheme S2). Recently, some of us have shown that **1** is formed from the diethyl (6-hydroxy-3-oxo-3*H*-xanthen-9-yl)methyl phosphate·2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) complex (**2**) upon irradiation at 520 nm (Scheme 1).[11] We further optimized and scaled up this photochemical procedure to produce tens of milligrams of **1** in high purity (Supporting Information).

Scheme 1. Synthesis of **1** (the incorporation of ^{18}O from water is shown in red)[11]



Scheme 2. Four Acid–Base Forms of **1** and the Corresponding p$K_{a,c}$ Values



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Four pH-dependent forms of **1** (**1a**–**d**; Scheme 2) and the corresponding p$K_{a,c}$ values were determined spectrometrically in aq buffer solutions ($K_{a,c}$ are concentration quotients at ionic strength $I \approx 0.1$ M; see Supporting Information and Figures S14–S15; the zwitterionic form of **1c** was predicted to be lower in energy (DFT, ~5 kcal mol^{-1}) than the corresponding charge-neutral tautomer). A dianion form **1a** (λ_{max} = 488 nm, Figure 1) is present at physiological pH (7.4) at >90%. Spectroscopic properties of **1** in methanol (Figure S13) are similar to those in an aq solution. The fluorescence quantum yield in aq buffer at pH = 7.4 was found to be relatively high (0.39 ± 0.03; λ_{em} ~530 nm; the single-exponential fluorescence lifetime is τ = 2.43 ± 0.08 ns; Figure 1; Table S1). The compound is stable in aq buffer at pH = 7.4 in the dark at 4 °C for at least a month.

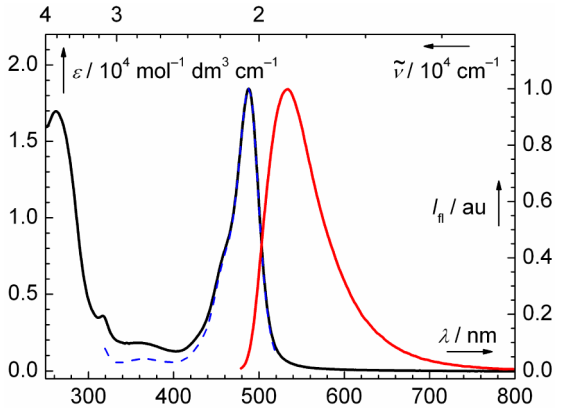
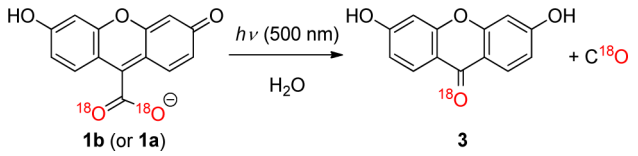


Figure 1. Absorption (black solid line), normalized emission (red solid line), and excitation (blue dashed line) spectra of **1** ($c \approx 1 \times 10^{-5}$ M) in 0.1 M aq phosphate buffer at pH = 7.4.

Photochemistry. Irradiation of **1** in water, methanol, and their mixtures at 500 nm gave an exclusive and isolable product, 3,6-dihydroxy-9*H*-xanthen-9-one (**3**, Scheme 3). The decomposition quantum yield (Φ) of **1**, determined using **2** as an actinometer,[11] was (6.8 ± 3.0) × 10^{-4} in aq phosphate buffer (pH = 7.4, I = 0.1 M; **1a** was the major (>97%) light-absorbing form present; see Figures S14 and S15). A higher Φ by a factor of ~6 ((3.9 ± 1.3) × 10^{-3}) was obtained at pH = 5.7, at which the monoanion **1b** and the dianion **1a** possess an equal absorbance at the excitation wavelength (Figures S14 and S15; the spectra of pure forms were obtained by the single value decomposition analysis; see Supporting Information). **3** was the sole photoproduct found at both pH's. The product of the molar absorption coefficient and the quantum yield, εΦ, which is proportional to the extent of release,[7a] was relatively large (on the order of 1–10) at $\lambda_{irr} \approx 500$ nm and pH = 7.4 due to large molar absorption coefficients of the corresponding forms. Therefore, the phototransformation of **1** was fast even when LEDs were used as an irradiation source.

Using the deconvoluted spectra (Figure S15) and the observed quantum yields at two different pH's (5.7 and 7.4),

Scheme 3. Photochemistry of **1a** or **b** at pH 5.7−7.4 (isotopically labeled ^{18}O is shown in red; the presence of ^{18}O in CO is only a presumption)



the decomposition quantum yield of the individual form **1b** was estimated to be higher by approximately 1 order of magnitude compared to that of **1a**, provided that Φ for each of the species is not affected by pH in this pH range.

The reaction efficiency at pH = 7.4 was not affected by the presence of oxygen. Thus either a triplet state was not involved or its lifetime was too short.

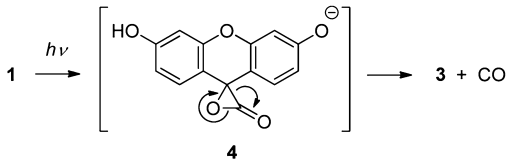
In contrast, an undetermined product with a λ_{max} of 430 nm (Figure S21) was formed in an aq solution at pH = 9.5 (the dianion **1a** was present exclusively) probably via a new concomitant (photo)reaction at such high hydroxide ion concentrations. Practically no photochemistry was observed at pH = 4.5, at which **1c** was the major absorbing species. **1** precipitated at pH = 2.5; thus the quantum yield could not be determined.

As a result, we conclude that both **1a** and **1b** are the only reactive species which undergo the phototransformation shown in Scheme 3 in the pH range 5.7−7.4.

Formation of two plausible gaseous side photoproducts, carbon monoxide and carbon dioxide, was considered. Irradiation of isotopically labeled **1** ($-C^{18}O_2H$ in the C9-position), prepared photochemically from **2** in $D_2{}^{18}O$ (Scheme 1),[11] in $H_2{}^{16}O$-based buffer (pH = 7.4; Scheme 3) gave **3** possessing the $C{=}^{18}O$ group (Figure S23). No isotopic incorporation to **3** occurred when **1** with the $-C^{16}O_2H$ group was irradiated in $D_2{}^{18}O$ (Figure S22). These experiments thus ruled out the direct involvement of the solvent in the phototransformation and suggested that carbon monoxide is the second photoproduct, most probably containing the oxygen atom from the parent carboxylic moiety (thermal decomposition of **1** leads to decarboxylation; Scheme S1).

Photorelease Mechanism. Based on the results of our isotopic labeling experiments, we hypothesized that the α-lactone **4**, which would further decarbonylate to form **3** (Scheme 4), might be formed as a primary product. It is known that α-lactones (oxiranones) are short-lived intermediates[12] that decompose efficiently by decarbonylation; the most stable known α-lactone has a half-life of ~8 h at 24 °C.[13] Our TD-DFT calculations showed that vertical excitation of **1** at the wavelengths of irradiation used (~500 nm) populates the lowest singlet excited state (S_1) for both the **1a** and **1b** forms (Tables S3−S5 and Figure S1). The relaxed S_1 potential energy surface (PES) scan along the coordinate of the C9−O bond length starting from the S_1 energy minimum of both forms led to an intermediate similar to **4** that was, however, > 40 kcal mol^{-1} higher in energy (Figure S2). The calculations did not indicate involvement of a conical intersection along the scanned coordinate, and we were unable to locate any local minimum for **4** on the ground state PES using various methods (Supporting Information). It is in agreement with the fact that α-lactone formation is favored only in systems that possess strong electron-withdrawing groups.[14] In addition, a ground-state transition state that connects **1** to both **3** and CO was found (Figure S3), but its high energy (> 50 kcal mol^{-1}) prevents a spontaneous decarbonylation of **1** at 20 °C. We also could not find a transition state for the CO release on the triplet hypersurface. Although our DFT calculations did not provide any evidence that the process involves **4**, its intermediacy should not be ruled out. Additional experiments and theoretical multiconfigurational models must be employed to fully understand the CO photorelease mechanism from **1**.

Scheme 4. Formation of a Putative Intermediate **4**



CO Trapping with Hemoglobin. A fast and sensitive method for determination of CO present in blood[15] or photoreleased from CORMs[16] often involves its complexation with hemoglobin (Hb) to form carboxyhemoglobin (COHb). In this work, an aqueous solution of uncomplexed Hb (Fe^{II}) was prepared by reduction of bovine methemoglobin (MetHb, Fe^{III}; $c = 2.3 \times 10^{-5}$ M) by sodium dithionite.[17] It was subsequently mixed with a solution of **1** ($c = 1.3 \times 10^{-4}$ M in 0.1 M aq phosphate buffer, pH = 7.4, purged with N_2), and **1** was irradiated at 503 ± 15 nm until complete conversion of Hb to COHb was observed. Formation of COHb was followed by absorption spectroscopy (Figure 2), although specific fluorescence signals of both **1** and **3** also allowed monitoring the course of the reaction. The distinct absorption characteristics of all species involved, **1a** (λ_{max} = 488 nm), Hb (λ_{max} = 405 nm), and COHb (λ_{max} = 419 nm) (Figure S17), therefore provide unique advantages for simultaneous observation of the CO complexation by using a

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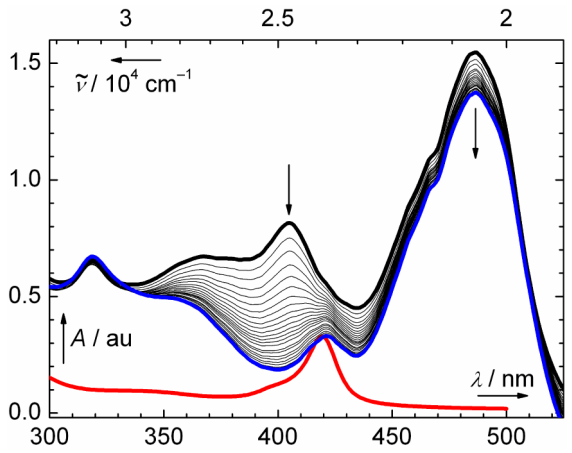

Figure 2. Absorption spectra (black lines) measured following irradiation of **1** ($c \approx 1.3 \times 10^{-4}$ M; the total irradiation time was 4.6 h) in the presence of MetHb ($c \approx 2.3 \times 10^{-5}$ M) and $Na_2S_2O_4$ ($c = 2.5 \times 10^{-5}$ M) in 0.1 M aq phosphate buffer at pH = 7.4 purged with N_2 at 503 ± 15 nm. The initial (black bold line) and final (blue bold line) spectra are highlighted. The spectrum of pure COHb formed from Hb and CO dissolved in water (red line) is shown for comparison.

selective excitation of the photoCORM **1** without spectral interference of the present hemoglobin derivatives.

In conclusion, 6-hydroxy-3-oxo-3*H*-xanthene-9-carboxylic acid (**1**) is the first representative of a transition-metal-free carbon monoxide releasing molecule activatable by visible light (photoCORM) that allows precise spatiotemporal control over the CO release in the presence of hemoglobin. Its favorable spectroscopic properties, good aqueous solubility, and transformation to a noninterfering photoproduct project possible applications in biology and medicine.

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Supporting Information Available. Materials and methods; synthesis and photophysical properties of the compounds; determination of pK_a of **1**; trapping of CO by hemoglobin; quantum chemical calculations; NMR, HRMS, UV−vis, and fluorescence data of new compounds.This material is available free of charge via the Internet at http://pubs.acs.org.

The authors declare no competing financial interest.