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CHEMICAL AND ENZYMATIC TRIGGERING OF 1,2-DIOXETANES. 3: ALKALINE PHOSPHATASE-CATALYZED CHEMILUMINESCENCE FROM AN ARYL PHOSPHATE-SUBSTITUTED DIOXETANE

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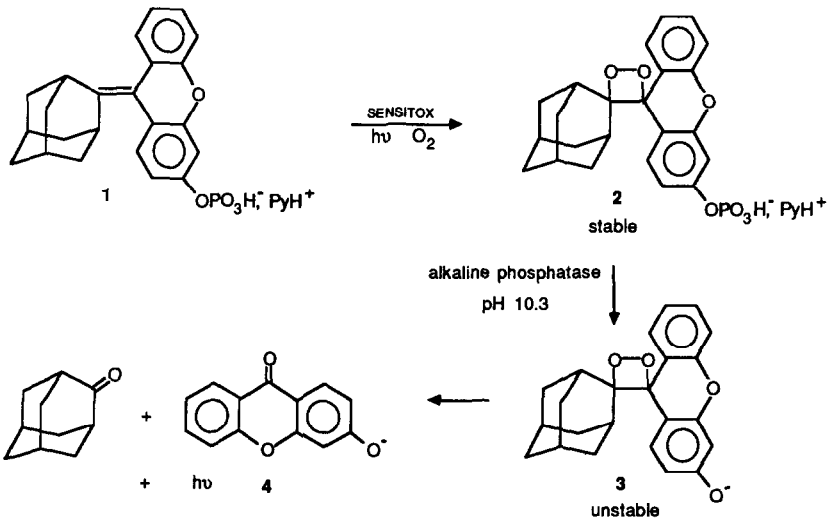
Abstract: We describe herein the preparation of an aryl phosphate-substituted 1,2-dioxetane and the enzymatic cleavage of this chemiluminescent dioxetane in aqueous buffer with alkaline phosphatase.

Biological assays involving alkaline phosphatase have utilized a wide variety of substrates which develop a color[1] (chromogenic) or become fluorescent[2] (fluorogenic) upon reaction with the enzyme. As part of our investigation of chemical and enzymatic triggering of 1,2-dioxetanes,[3] we have developed the first example of a chemiluminescent or luminogenic substrate for this enzyme.[4] Utilization of these peroxides in biological systems requires dioxetanes that are thermally stable at ambient temperature, do not undergo rapid spontaneous decomposition in the aqueous buffers, and yield upon enzymatic removal of the appropriate functional group an unstable aryloxide form which provides the luminescence. The phosphate-substituted dioxetane **2** meets these requirements and undergoes cleavage with emission of light in aqueous buffer at pH 10.3 in the presence of alkaline phosphatase.

3-Phosphate-9H-xanthen-9-ylideneadamantane, monopyridinium salt (**1**) was prepared from 2-adamantanone and 3-hydroxy-9H-xanthen-9-one.[5] Dioxetane **2** was produced by photooxygenation of **1** in dioxane using polymer-bound Rose Bengal[6] (SENSITOX I) and a 1000-W high-pressure sodium lamp. After irradiation for 30 min, the sensitizer was removed by filtration to provide a 2.5 x 10^{-4} M stock solution of **2**.[7] TLC and NMR showed the absence of starting alkene and carbonyl cleavage products. Like the aryl adamantyl dioxetanes described in the preceding paper, adamantyl xanthenyl dioxetanes also exhibit a high degree of thermal stability.[8] For example, thermolysis of **2** in *o*-xylene at 95 °C yields chemiluminescence with a half-life of 2.5 h.



The phosphatase triggering experiments were conducted using the phosphate-protected dioxetane **2** at pH 10.3 in 0.75 M 2-amino-2-methyl-1-propanol buffer. Alkaline phosphatase from bovine intestinal mucosa was purchased from Sigma Chemical Co. as a suspension of 5.3 mg of protein (1100 units/mg protein) per mL in 3.2 M $(NH_4)_2SO_4$ solution. A 50 μL aliquot (0.013 μmol) of the phosphate-dioxetane stock solution was added to 3 mL of the buffer at 37 °C to give a final dioxetane concentration of 4.2×10^{-6} M. Injection of 1 μL (final conc of protein = 1.8 μg/mL) of alkaline phosphatase to the solution resulted in a burst of chemiluminescence that decayed over a period of 3 min. The total light emission was found to be linearly dependent on the dioxetane concentration. The rate of decay of the emission is a function of enzyme concentration while the total light emission is independent of the enzyme concentration (Figure 1). Under these conditions the background luminescence from slow hydrolysis of **2** in the buffer was only 0.2% of that produced by the enzymatic process.

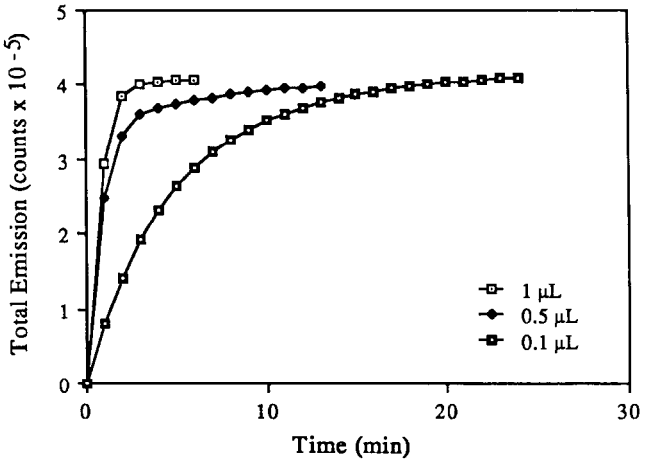


Figure 1. Plot of total chemiluminescence from alkaline phosphatase triggering of dioxetane **2** at pH 10.3 with 1, 0.5, and 0.1 μL of phosphatase suspension.

Several control experiments have shown that this luminescence is the result of an enzyme-catalyzed reaction of **2**: 1.) Catalysis of the dioxetane decomposition by the solution in which the enzyme is suspended was ruled out since the addition of 10 μL of 3.2 M $(NH_4)_2SO_4$ to 3 mL of buffer solution containing 50 μL of dioxetane stock at 37 °C produced no chemiluminescence. Subsequent addition of 1 μL of phosphatase produced chemiluminescence with the typical decay curve. 2.) In experiments where 50 μL of dioxetane stock in 3 mL of buffer solution was triggered with 10 μL of phosphatase at 25, 37, and 50 °C, the maximum light intensity and the rate of chemiluminescence decay both increased with increasing temperature. 3.) The addition of the monosodium salt of 1-naphthyl phosphate, a known alkaline phosphatase substrate, caused a reversible inhibition of the chemiluminescence. For example, a solution of 200 μL of dioxetane stock in 3 ml of buffer solution at 25 °C was treated with 10 μL of phosphatase to initiate chemiluminescence. At the point of maximum light intensity, 50 μL of a 0.01 M aqueous solution of 1-naphthyl phosphate was added. A rapid decrease in the light intensity was observed followed by a rapid restoration to the original intensity. 4.) The addition of the known enzyme inhibitor, sodium dodecyl sulfate (SDS), to a solution of 200 μL of dioxetane stock, 3 mL of buffer solution, and 10 μL of phosphatase at 25 °C caused an irreversible decrease in the light intensity. The addition of sufficient SDS resulted in the complete inhibition of chemiluminescence.

Many dioxetanes are known to be decomposed via non-luminescent pathways by amines[9] and metal ions.[10] Therefore, a series of experiments was performed to determine the stability of the phosphate-dioxetane **2** in the amine buffer over an extended period. A comparison was made of the total light emitted in two experiments from 50 μL of dioxetane stock in 3 mL of buffer solution: a) injection of 10 μL of enzyme immediately after addition of the dioxetane to the buffer and b) injection of the enzyme 30 min after the dioxetane was added. If the dioxetane decomposed appreciably in the buffer, then the total emission of the experiment where the dioxetane was exposed to the buffer for 30 min would be decreased. At 25 °C, there was no change in the total light emitted while at 37 °C, the emission was decreased by only 6% after the 30 minute delay.

The chemiluminescence spectrum for the phosphatase-catalyzed decomposition of **2** was obtained at room temperature in the buffer solution (Figure 2).[11] A comparison of this chemiluminescence spectrum with the fluorescence spectrum of the spent reaction mixture and the fluorescence spectrum of **4** in the buffer indicates that the emission is initiated by the enzymatic cleavage of the phosphate group in dioxetane **2** to generate singlet excited **4**.

The chemiluminescence quantum yield for the phosphatase triggering of **2** is 1.4×10^{-6} using luminol as a light standard.[12] The fluorescence quantum yield for the xanthone cleavage product **4** in the buffer is 0.46 so that the efficiency for the formation of the singlet excited state of **4** in the enzymatic process is 3×10^{-4}%. That this low value for the chemiexcitation efficiency is not due to quenching of **4** by the protein is shown by the fact that base-triggering of the corresponding dioxetane derived from 3-hydroxy-9H-xanthen-9-ylideneadamantane also exhibits a chemiluminescence quantum yield of only 1.2×10^{-6} both in the buffer and in dilute NaOH solution (pH 10).

As demonstrated in the preceding paper, the chemiexcitation efficiency of these dioxetanes can be dramatically altered by relatively simple changes in the structure of the aryloxide group. We are, therefore, currently investigating a variety of other phosphate-substituted dioxetanes in order to gain an understanding of the factors that determine the efficiency of the excitation process in both chemical and enzymatic triggering. The present results do, however, illustrate the potential sensitivity of this methodology for new types of biological assays involving appropriately substituted dioxetanes and enzyme triggering.

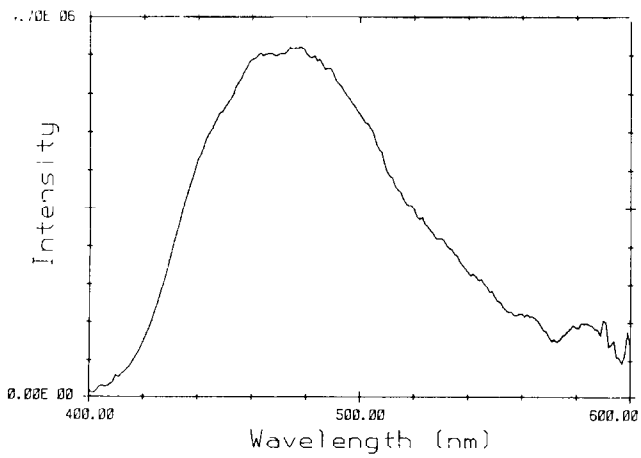


Figure 2. Chemiluminescence spectrum from alkaline phosphatase triggering of dioxetane **2** in pH 10.3 buffer at room temperature.

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3. See the preceding paper and: Schaap, A. P.; Handley, R. S.; Giri, B. P. *Tetrahedron Lett.*, in press.

4. Luminol derivatives and oxalate esters have been used with peroxidases or oxidases for chemiluminescent biological assays. See, for example, (a) Schroeder, H. R.; Yeager, F. M. *Anal. Chem.* **1978**, *50*, 1114. (b) Arakawa, H.; Maeda, M.; Tsuji, A. *Anal. Biochem.* **1979**, *79*, 248. (c) Arakawa, H.; Maeda, M.; Tsuji, A. *Ibid.* **1985**, *31*, 430. However, these systems differ from the present case in that alkaline phosphatase acts directly on the luminogenic substrate **2**.

5. The preparation of **1** will be described in a subsequent paper: ^{1}H NMR ($CDCl_3$) δ 1.8-2.1 (m, 12H), 3.42 (s, 1H), 3.50 (s, 1H), 7.1-7.4 (m, 7H), 8.1 (m, 2H), 8.55 (t, 1H, J=7.6 Hz), 8.8 (m, 2H); ^{13}C NMR ($CDCl_3$) δ 27.68, 32.53, 32.59, 36.77, 39.33, 109.17, 114.57, 115.22, 116.42, 123.15, 125.81, 126.04, 127.16, 127.47, 127.57, 128.46, 141.53, 146.63, 147.86, 147.94, 154.53, 155.79; ^{31}P NMR ($CDCl_3$) δ 6.12 relative to external H_3PO_4.

6. (a) Schaap, A. P., Thayer, A. L., Blossey, E. C., Neckers, D. C. *J. Am. Chem. Soc.* **1975**, *97*, 3741. (b) Schaap, A. P., Thayer, A. L., Zaklika, K. A., Valenti, P. C. *Ibid.* **1979**, *101*, 4016.

7. Dispiro[adamantane-2',3-[1,2]dioxetane-4,9"-(3-phosphate-9H-xanthene)] (**2**): ^{1}H NMR ($CDCl_3$) δ 1.5-2.1 (m, 12H), 2.33 (s, 1H), 2.43 (s, 1H), 7.2-7.5 (m, 5H), 7.8 (m, 2H), 8.15 (dd, 1H, J=7.8, 1.5 Hz), 8.24 (dd, 1H, J=8.4, 1.1 Hz), 8.3 (m, 1H), 8.4 (m, 2H); ^{13}C NMR ($CDCl_3$) δ 25.16, 25.34, 31.53, 31.59, 32.63, 32.96, 33.06, 35.78, 84.36 (dioxetane ring carbon), 96.81 (dioxetane ring carbon), 108.60, 115.24, 116.03, 116.43, 120.79, 120.99, 123.69, 128.22, 130.04, 130.15, 135.23, 150.04, 150.19, 150.81, 151.99; ^{31}P NMR ($CDCl_3$) δ 6.03 relative to external H_3PO_4.

8. For other adamantyl-substituted dioxetanes, see (a) Wieringa, J. H.; Strating, J.; Wynberg, H.; Adam, W. *Tetrahedron Lett.* **1972**, 169. (b) Turro, N. J.; Schuster, G.; Steinmetzer, H. -C.; Faler, G. R.; Schaap, A. P. *J. Am. Chem. Soc.* **1975**, *97*, 7110. (c) McCapra, F.; Behesti, I.; Burford, A.; Hann, R. A.; Zaklika, K. A. *J. Chem. Soc., Chem. Commun.* **1977**, 944. (d) Adam, W.; Encarnacion, L. A. A.; Zinner, K. *Chem. Ber.* **1983**, *116*, 839.

9. (a) Wilson, T. *Int. Rev. Sci.: Phys. Chem., Ser. Two.* **1976**, *9*, 265.

10. (a) Wilson, T.; Landis, M. E.; Baumstark, A. L.; Bartlett, P. D.. *J. Am. Chem. Soc.* **1973**, *95*, 4765. (b) Bartlett, P. D.; Baumstark, A. L.; Landis, M. E. *Ibid.* **1974**, *96*, 5557.

11. Chemiluminescence spectra were acquired with a Spex Fluorolog spectrofluorometer. Correction for the decay of the chemiluminescence intensity during the wavelength scan was made by accumulating the spectrum in a ratio mode using an auxiliary detector to measure the total signal as a function of time.

12. (a) Lee, J.; Seliger, H. H. *Photochem. Photobiol.* **1972**, *15*, 227. (b) Michael, P. R.; Faulkner, L. R. *Anal. Chem.* **1976**, *48*, 1188. Quantum yields were determined using a luminometer constructed in our laboratory with an RCA A-31034A gallium-arsenide PMT cooled to - 78 °C and Ortec photon-counting electronics.

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