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CHEMICAL AND ENZYMATIC TRIGGERING OF 1,2-DIOXETANES. 1: ARYL ESTERASE-CATALYZED CHEMILUMINESCENCE FROM A NAPHTHYL ACETATE-SUBSTITUTED DIOXETANE‡

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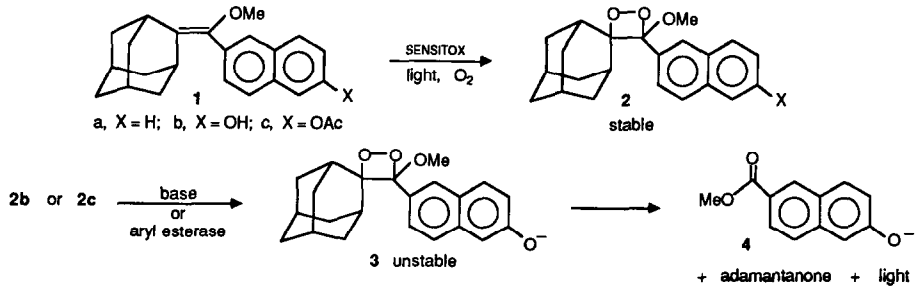
Abstract: A thermally stable 1,2-dioxetane bearing a naphthyl acetate group is enzymatically cleaved in aqueous buffer to generate chemiluminescence at ambient temperature.

More than 350 papers concerning 1,2-dioxetanes[1] have appeared since the first reports of the chemical[2] and photochemical[3] preparation of these novel peroxides. Dioxetanes exhibit a wide range of thermal stabilities. For example, the sterically hindered dioxetane derived from adamantylideneadamantane[4] has a half-life at 25 °C of over 20 years and a melting point of 174 °C while dioxetanes obtained from enamines[5] decompose rapidly (and sometimes explosively) below 0 °C. The differences in the properties of these two types of dioxetanes arise because of two competing mechanisms for decomposition. Stable dioxetanes cleave by a process that requires 25 - 37 kcal for homolysis of the O-O bond and formation of an intermediate biradical.[1] An alternative mechanism involving intramolecular electron transfer is available to dioxetanes bearing substituents with low oxidation potentials.[5d,6]

In 1982 we demonstrated that chemiluminescence from a dioxetane bearing a phenolic substituent could be triggered in organic solvents by the addition of base.[7] Deprotonation generates an unstable phenoxide-substituted dioxetane which decomposes 4.4 x 10^6 times faster than the protonated form. We have now developed several other methods for inducing the cleavage of thermally stable dioxetanes and in this report provide the first example of *enzymatic* triggering of a chemiluminescent dioxetane. The present case utilizes aryl esterase to catalyze the cleavage of a naphthyl acetate-substituted dioxetane in aqueous buffers at ambient temperature.

Dioxetanes **2a-c** were prepared by photooxygenation of the corresponding alkenes[8] in CH_2Cl_2 using polymer-bound Rose Bengal (SENSITOX I), a 1000-W high-pressure sodium lamp, and methods previously described.[9] After 15 - 30 min irradiation the sensitizer was removed by filtration and the solvent evaporated under vaccum. Recrystallization of the material from pentane/ether gave the dioxetanes in 75 - 95 yields.[10]



‡The senior author wishes to dedicate this paper to Professor Paul D. Bartlett upon the occasion of his retirement at Texas Christian University.

Table I. Activation Parameters and Rates of Decomposition for 1,2-Dioxetanes 2a-c in o-Xylene.

substituent X	E_a(kcal/mol)[a]	log A	k(sec^{-1}) at 25 °C	half-life at 25 °C[b]
H	29.7	13.2	3.17×10^{-9}	6.9 years
OH	29.7	13.3	3.83×10^{-9}	5.1 years
OAc	32.5	14.9	1.19×10^{-9}	18.5 years

(a) Rates showed variations of less than 3% and gave Arrhenius plots with $r > 0.99$. Values of E_a are ±1kcal/mol.
(b) Calculated from the Arrhenius plots.

Rate constants for the thermal decomposition of dioxetanes **2a-c** were obtained at 80 to 120 °C from measurements of the decay of chemiluminescence intensity of 10^{-4} M solutions in *o*-xylene. The incorporation of the adamantyl group in **2a-c** affords dioxetanes of remarkably high thermal stability (Table I),[11,12] The decomposition of **2b** can, however, be triggered by treatment with excess potassium *tert*-butoxide in o-xylene to give an intense blue chemiluminescence which decays with a half-life of approximately 20 s at 25 °C. Experiments with **2b** in methanol using KOH also resulted in chemiluminescence with similar decay rates. Comparison of the spectrum of the base-induced chemiluminescence with the fluorescence of anion **4** under identical conditions demonstated that the luminescence is derived from chemiexcited **4**.

Enzymatic triggering experiments were conducted with the acetyl-protected dioxetane **2c** at pH 7.6 in 0.05 M phosphate and 0.02 M Tris buffers. A 2 mM stock solution of **2c** in 2-methoxyethanol was prepared. Aryl esterase (carboxylic ester hydrolase (E-3128)) from porcine liver was purchased from Sigma Chemical Co. as a suspension of 11 mg of protein (260 units/mg) per mL in 3.2 M $(NH_4)_2SO_4$ solution. When a 150 μL (0.3 μmol) aliquot of the stock solution of **2c** was added to 3 mL of either buffer solution at 25 °C, no emission was detected. However, injection of 1 μL (0.26 units, final conc of protein = 3.6 μg/mL) of aryl esterase to the stirred solution generated chemiluminescence. The time required for one-half of the total light emission was found to be 7 min in both buffers. A similar time course for the consumption of **2c** was found when the reaction was followed by UV spectroscopy. Additional experiments showed that total light emission is linearly dependent on dioxetane concentration over the range of 10^{-4} to 10^{-7} M. The rate of decay of the emission is a function of enzyme concentration while total light emission is independent of enzyme concentration (Figure 1).

That this chemiluminescence is due only to an enzyme-catalyzed hydrolysis of acetate **2c** is demonstrated by the following series of experiments: 1.) Denaturing the enzyme by heating 1 μL in 3 mL of Tris buffer to 90 °C and

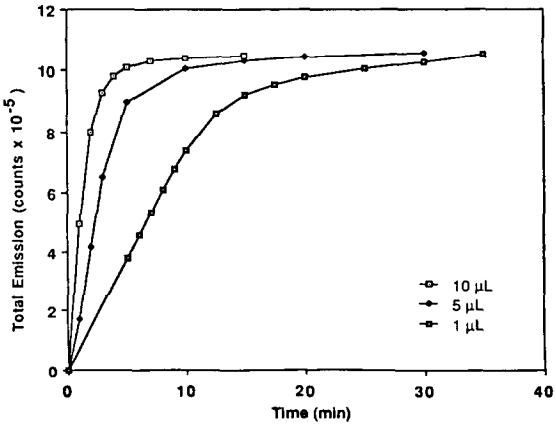


Figure 1. Plot of total chemiluminescence from esterase triggering of dioxetane 2c in Tris buffer with 1, 5, and 10 μL of esterase suspension.

cooling to 25 °C resulted in no chemiluminescence when an aliquot of the dioxetane stock solution was subsequently added. Addition of untreated enzyme preparation to this solution again produced light. 2.) In experiments where 150 μL of dioxetane stock in 3 mL of Tris buffer was triggered with 1 μL of enzyme at 25, 37 and 50 °C, the maximum light intensity (I_{max}) and the rate of decay both increased with increasing temperature. 3.) Addition of the known enzyme inhibitor, sodium dodecyl sulfate (SDS) at I_{max} caused an irreversible decrease in the intensity. The emission could be totally extinguished by addition of sufficient SDS. The decrease in light emission is not due to photophysical quenching of the excited state since thermal decomposition in the same solvent system with SDS at elevated temperatures results in readily detectable chemiluminescence. 4.) Addition of esterase substrates (α-naphthyl and β-naphthyl acetates) caused a rapid decrease in light intensity followed by restoration of the original intensity within 1 min. 5.) Sequential injection of ten identical aliquots of the dioxetane stock solution when light emission had stopped resulted in identical chemiluminescence curves, both in I_{max} and time for complete decay of the signal.

Many dioxetanes are known to be destroyed via non-luminescent pathways by amines[1a] and metal ions.[13] Therefore, a series of experiments was performed to assess the stability of **2c** in the buffers over the time course of a typical run. A comparison was made between I_{max} of two experiments with the enzyme added immediately after the dioxetane or after a 30 min delay. If the dioxetane were decomposing in the buffer, then I_{max} of the run where the dioxetane was exposed to the buffer for 30 min would be lower. In phosphate buffer at 25 °C, no decrease in I_{max} was observed after the 30 min delay with only a 12% decrease observed in Tris buffer.

The chemiluminescence spectrum for the enzyme-catalyzed decomposition of **2c** in Tris buffer at ambient temperature matches the fluorescence spectrum of methyl 6-hydroxy-2-naphthoate in the buffers and in strongly basic solution (Figure 2).[14] The spectrum of the chemiluminescence from the spontaneous decomposition of the hydroxy-dioxetane **2b** under the same conditions was also identical. These findings demonstrate that rate-limiting enzymatic hydrolysis of the acetate group in dioxetane **2c** generates the unstable dioxetane **3** which subsequently yields singlet excited **4**.

These studies have shown for the first time that thermally stable 1,2-dioxetanes can be enzymatically triggered at ambient temperatures to generate chemiluminescence in the presence of aqueous buffers and proteins. Subsequent papers will report the chemiexcitation efficiencies for **2b** and **2c**. We will also describe the triggering of appropriately substituted dioxetanes by other enzymatic systems including alkaline phosphatase.

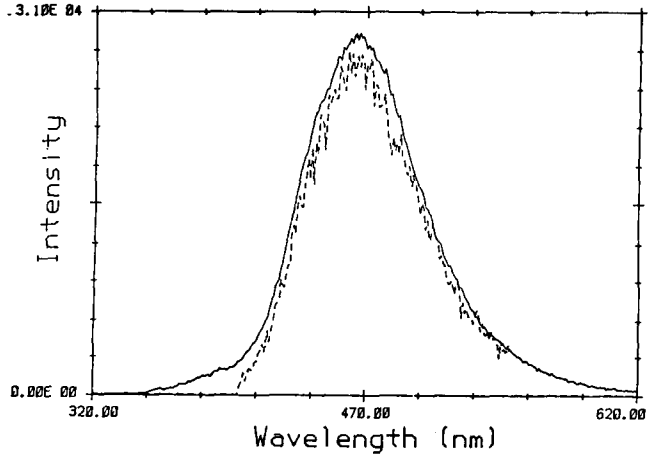


Figure 2. Chemiluminescence spectrum from esterase triggering of dioxetane 2c in Tris buffer at room temperature (---). Fluorescence spectrum of 4 under the same conditions (—).

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(10) **2a**: mp 116 °C (dec); ^{1}H NMR δ 0.9-2.0 (m, 12H), 2.22 (s, 1H), 3.11 (s, 1H), 3.24 (s, 3H), 7.0-8.3 (m, 7H); ^{13}C NMR δ 25.94, 26.07, 31.60, 31.72, 32.31, 33.08, 33.23, 34.88, 36.42, 50.00, 95.60, 112.33, 125.21, 126.47, 127.02, 127.63, 127.91, 128.67, 129.41, 132.13, 132.85, 133.61. **2b**: mp 107-9 °C (dec); ^{1}H NMR δ 0.9-2.0 (s, 12H), 2.20 (s, 1H), 3.09 (s, 1H), 3.24 (s, 3H), 7.1-7.9 (m, 6H); ^{13}C NMR δ 25.91, 26.03, 31.58, 31.68, 32.33, 33.02, 33.22, 34.84, 36.40, 49.99, 95.77, 109.37, 112.48, 118.35, 125.85, 126.39, 128.22, 129.29, 129.74, 130.67, 134.95, 154.55. **2c**: mp 122-4 °C (dec); ^{1}H NMR δ 0.9-2.0 (m, 12H), 2.18 (s, 1H), 2.37 (s, 3H), 3.10 (s, 1H), 3.23 (s, 3H), 7.27 (d, 1H, J = 2.1 Hz), 7.59 (d, 1H, J = 2.1 Hz), 7.84 (d, 1H, J = 9.0 Hz), 7.95 (d, 1H, J = 9.0 Hz); ^{13}C NMR δ 21.12, 25.93, 26.06, 31.60,31.73, 32.29, 33.09, 33.24, 34.89, 36.39, 49.88, 95.57, 112.19, 118.44, 121.82, 125.96, 127.70, 129.22, 130.21, 130.89, 132.19, 134.05, 149.33, 169.51.

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(14) Fluorescence experiments with methyl 6-hydroxy-2-naphthoate in water have shown that the singlet excited state undergoes deprotonation and emission from the anion **4**.

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