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SYNTHESIS AND ENZYMATIC HYDROLYSIS OF A LIGHT-EMITTING SUBSTRATE FOR PHOSPHOTRIESTERASE

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Abstract: The diethylphosphate derivative of 4-methoxy-4-(3-hydroxyphenyl)spiro[1,2-dioxetane-3,2'-adamantane] was synthesized. The K_m and V_{max} values for the enzymatic hydrolysis by phosphotriesterase were 0.091 mM and 0.15 sec^{-1}, respectively. The enzyme-catalyzed hydrolysis was accompanied by the emission of visible light.

The bacterial phosphotriesterase from *Pseudomonas diminuta* has a broad substrate specificity for the hydrolysis of organophosphate nerve agents.[1] Extensive mechanistic and structural studies with this protein have been conducted because of the potential utility of this enzyme for the rapid detoxification of organophosphate insecticides and chemical warfare agents.[2] It has previously been reported that substituted adamantyl 1,2-dioxetanes are stable at room temperature. These molecules have proven to be quite useful for the assay and localization of minute quantities of various enzymes because of the ability of these compounds to emit visible light upon enzymatic cleavage of a suitable protecting group.[3] Substrates of this type for the bacterial phosphotriesterase would be beneficial for the rapid screening of large numbers of random and site-directed mutants with altered kinetic properties. This report focuses on the synthesis and enzymatic properties of a dioxetane with a diethylphosphate protecting group. Chemiluminescence can be triggered from this adamantyl-stabilized 1,2-dioxetane by enzymatic cleavage of the diethyl phosphate group to the unstable aryloxide intermediate and rearrangement of the dioxetane ring to yield the exited singlet state ester as summarized in Figure 1.[4]

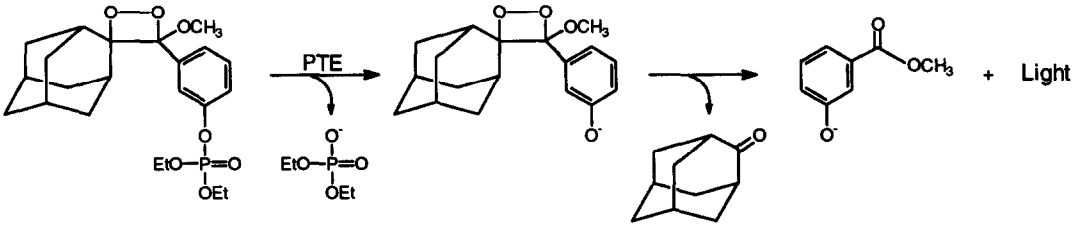

Figure 1. Enzymatic hydrolysis of diethylphosphate derivative.

Experimental Methods

General. All of the chemicals were purchased from Aldrich except for the polymer-bound Rose Bengal (SENSITOX I), which was obtained from Dyetel and Lumi-Phos™ 530, which was purchased from Boehringer. The phosphotriesterase used for these experiments was purified and reconstituted with two equivalents of Co^{2+} as previously described.[5]

Synthesis of Phosphotriester. The synthesis of [(3-hydroxyphenyl)methoxymethylene]adamantane (1) was accomplished according to the procedure of McMurry *et al.*[6] The compound was dissolved in pyridine and then slowly added to a cold mixture of diethyl chlorophosphate and pyridine at 5 °C. The reaction mixture was allowed to stir for 24 hours at room temperature. The crude product was extracted with CH_2Cl_2, washed with 10% $CuSO_4$, dried over $MgSO_4$, and evaporated to dryness. The phosphotriester (2) was purified by silica gel chromatography (20% EtOAc/n-hexane).[7] The dioxetane (3) was synthesized by photooxygenation of 2 in CH_2Cl_2 using polymer-bound Rose Bengal (SENSITOX I) and a 1000-W high pressure sodium lamp. After 20 minutes of irradiation at -78 °C, the sensitizer was filtered and the solvent was removed under vacuum. The residue was further purified by flash column chromatography (20% EtOAc/n-hexane) to give the final product.[8] All ^1H NMR, ^{13}C NMR, and mass spectral data were consistent with the expected structure of the product. The reaction scheme is summarized in Figure 2.

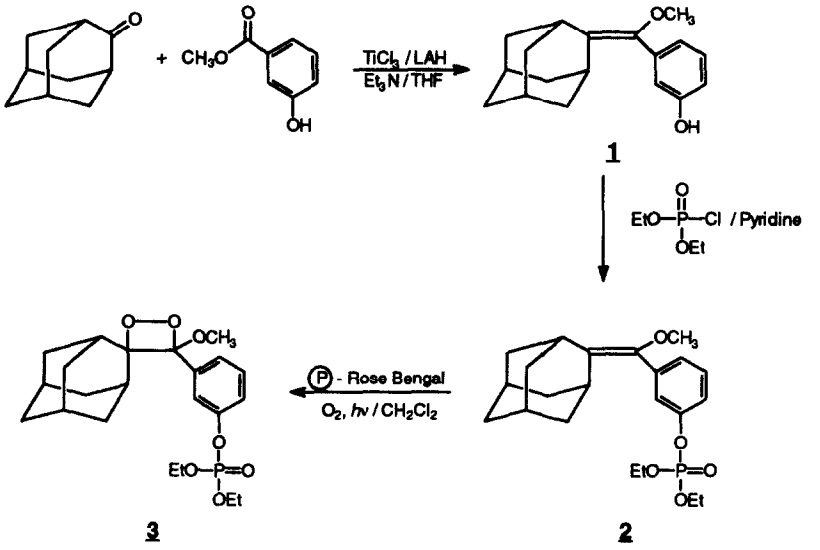

Figure 2. Synthesis of 4-methoxy -4-(3-diethylphosphatephenyl)spiro[1,2-dioxetane-3,2'-adamantane].

Kinetic Measurements. All kinetic measurements were performed as reported previously with a Gilford Model 260 spectrophotometer.[5] The kinetic parameters, V_{max} and K_m, for the hydrolysis of compound **3** with the phosphotriesterase were obtained by spectrophotometrically monitoring the formation of methyl 3-hydroxybenzoate at 250 nm using an extinction coefficient of 6 $mM^{-1} cm^{-1}$ at pH 10 in 50 mM CHES buffer. The kinetic data were fit to the following equation, where V_{max} is the maximal velocity, K_m is the Michaelis constant, and A is the initial substrate concentration.

$$v = V_m A / (K_m + A) \quad (1)$$

Measurement of Light. The chemiluminescence of the sample, after addition of enzyme, was measured with the aid of a homebuilt luminometer. The phosphotriesterase (2.3 nM) was incubated at 25 ^{0}C in 0.1M CHES buffer, pH 10, containing 15 % MeOH in a final volume of 300 μL. Various concentrations of compound **3** were added to this solution to initiate light emission. Light emission was enhanced by adding 0.23 mM cetyltrimethyl ammonium bromide and 7 μM fluorescein surfactant from the Lumi-Phos™ reagent.

Results and Discussion

Initial Rate Studies. For the enzymatic hydrolysis reaction of compound **3** with the Co-substituted phosphotriesterase, the values for K_m, and V_{max} were 0.091 mM and 0.15 sec^{-1}, respectively.

Light Production. The production of light from compound **3** after the addition of the phosphotriesterase was measured with the luminometer. The time courses for light emission with various concentrations of the triester are shown in Figure 3. Under these conditions the light intensity reaches a maximum in about 3 minutes and then gradually declines. The maximum signal intensity could be increased with higher levels of enzyme (data not shown) and the duration of light output could be extended with elevated levels of the fluorescein surfactant.

The dioxetane **3** is a reasonable substrate for the phosphotriesterase. The K_m exhibited by this compound is comparable to that previously obtained for the hydrolysis of paraoxon (~100 μM). However, the maximum velocity for hydrolysis of the P-O bond in this compound is about 4 orders of magnitude smaller than the hydrolysis of paraoxon with this enzyme. This rate reduction is due predominantly to the difference in the pK_a values of the leaving group phenol between the two substrates. Increased rates could be achieved by the synthesis of alternative substrates with electron withdrawing substituents on the aromatic ring. Nevertheless, measureable amounts of visible light are emitted coincident with the enzymatic hydrolysis of **3**. This compound

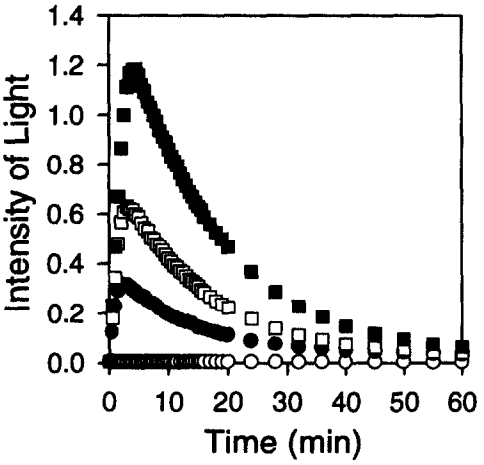

Figure 3. The light intensity of compound **3** upon addition of phosphotriesterase. The time course of the intensity of light is shown with various concentration of compound **3**: (○) 0 μM, (●) 42.6 μM, (□) 85.2 μM, and (■)170 μM. The unit for the intensity of light is given as an arbitrary value. Experimental details are described under Experimental Methods.

should prove useful in the assay of phosphotriesterase activity and may find some utility as a substitute for the galactoside and phosphate protected dioxetanes.

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References

1. Dumas, D. P.; Caldwell, S. R.; Wild, J.R.; Raushel, F. M. *J. Biol. Chem.* **1989**, *264*, 19659-19665
2. Caldwell, S. R.; Newcomb, J. R.;Schlecht, K. A.; Raushel, F. M. *Biochemistry*, **1991**, *30*, 7438-7444
3. Schaap, A. P.; Chen, T. S.; Handley, R. S.; DeSilva, R.; Giri, B. P. *Tetrahedron Lett.* **1987**, 1155-1158
4. Schaap, A. P.; Akhavan, H.; Romano, L. *J. Clin. Chem.* **1989**, *35*, 1863-1864
5. Omburo, G. A.; Kuo, J. M.; Mullins, L. S.; Raushel, F. M. *J. Biol. Chem.* **1992**, *267*, 13278-13283
6. McMurry, J. E.; Miller, D. D. *J. Am. Chem. Soc.* **1983**, *105*, 1660-1661
7. ^{1}H NMR (200 MHz, $CDCl_3$) δ 1.35 (6H, dt), 1.64-1.96 (12H, m), 2.65 (1H,s), 3.24 (1H, s), 3.30(3H, s), 4.23 (4H, q), 6.95-7.42 (4H, m), ^{13}C NMR (200 MHz, $CDCl_3$) δ 16.10, 28.19, 28.29,30.18, 32.13, 32.68, 37.08, 38.68, 39.09, 57.80, 64.57, 118.93, 120.76, 125.88, 129.16, 132.52, 137.25, 142.54, 150.58.
8. ^{1}H NMR (200 MHz, $CDCl_3$) δ 1.35 (6H, dt), 1.47-1.96 (12H, m), 2.15 (1H, s), 3.05 (1H, s), 3.22 (3H, s), 4.23 (4H, q), 7.25-7.60 (4H, m), ^{13}C NMR (200MHz, $CDCl_3$) δ 15.95, 25.86, 30.73, 31.55, 32.15, 32.81, 33.02, 34.62, 36.25, 49.88, 64.68, 95.45, 111.61, 121.29, 129.71, 137.02, 151.15. FAB mass spectrum : m/e 439 (M+H)

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