Direct Kinetic Evidence for the Formation of an Acylpyridinium Intermediate in Synthetic *p*-Nitrophenyl Esterase-Catalyzed Hydrolysis Reactions

Guang-Jia Wang and Wilmer K. Fife*

Department of Chemistry, Indiana University−Purdue University at Indianapolis, 402 N. Blackford Street, Indianapolis, Indiana 46202

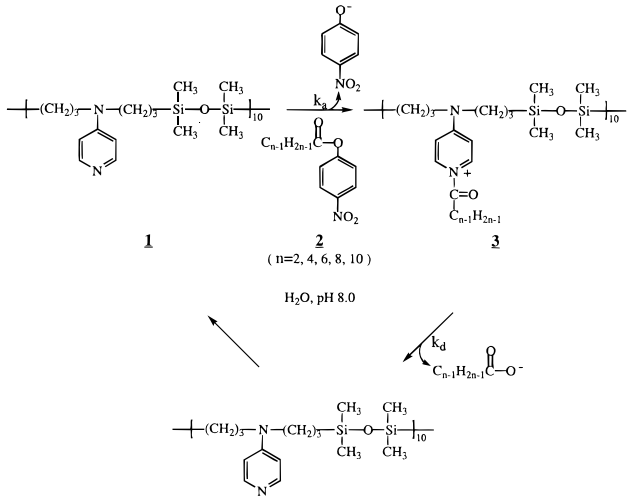
Received July 19, 1996; Revised Manuscript Received October 21, 1996[⊗]

ABSTRACT: The kinetics of the hydrolysis of *p*-nitrophenyl alkanoates **2** catalyzed by **1** were investigated in aqueous Tris buffer solution. Direct evidence for the existence of an *N*-acylpyridinium intermediate **3** was obtained for **1**-catalyzed hydrolysis of **2** ($n = 2-10$). Increase of the alkyl chain length leads to an increase in the acylation rate, which reaches a maximum for **2** ($n = 6$). The acylation rate then decreases progressively with further increases of alkyl chain length in substrate esters. The deacylation rate was also found to exhibit a maximum for the same substrate **2** ($n = 6$). These results are similar to those previously reported with cholesterol esterase as catalyst for the same hydrolysis reaction. The acylation reaction is first order in catalyst concentration and exhibits saturation kinetics at high substrate concentration in accordance with the Michaelis−Menten model for enzyme reaction kinetics.

Introduction

Since the discovery that 4-(dimethylamino)pyridine (DMAP) and its derivatives (DAAP) are remarkably powerful acyl-transfer catalysts,[1,2] a large research effort has been devoted to the syntheses of 4-(dialkylamino)pyridine-functionalized polymers (poly(DAAP)) in order to attain goals of mimicking enzymic efficiency and selectivity.[3−12] Most studies have been focused on the design of the active-site microenvironment of the synthetic catalysts to achieve the maximum catalytic activity by introducing a reactive group that can function in concert with a selective binding site to accelerate the desired chemical reaction.[7,8] A number of such polymers have been evaluated as supernucleophilic catalysts in the hydrolysis of *p*-nitrophenyl alkanoates **2** in aqueous and aqueous methanol solution.[3,7,8] Many of the studies have been promoted by an interest in modeling enzyme behavior as evidenced by enhanced levels of reactivity combined with expression of selectivity toward particular substrates.[8,13−15] The hydrolysis reactions catalyzed by these synthetic polymers have followed Michaelis−Menten kinetics and yielded other results commonly observed with enzymes. One of these polymers, **1**, containing the DAAP functionality and a bis(trimethylene)disiloxane group in its backbone structure exhibits enzyme-like substrate specificity in the solvolysis of **2** ($n = 2-18$).[8−12] This synthetic, oligomeric *p*-nitrophenyl esterase shows highest levels of activity toward **2** ($n = 14$) in 1:1 (v/v) aqueous methanol solution.[8] Despite intensive investigation of the catalytic properties of polymers with DAAP as the functional group, the mechanistic details of the catalysis remain obscure.[3,7,8] The mechanism for catalysis is assumed to involve attack by nucleophile **1** at the carbonyl group of substrate **2** and formation of an *N*-acylpyridinium intermediate **3** as in Scheme 1. Until recently,[16] no direct evidence for the existence of the putative *N*-acylpyridinium intermediate **3** had been obtained in DAAP-catalyzed hydrolysis reactions of **2**.[3,15] Previous efforts to document participation in nucleophilic catalysis by a DAAP-functionalized oligomer **1** in 1:1 methanol−water, phosphate buffer, were unsuccessful.[8]

Scheme 1



In the present paper, we report direct evidence for the formation of an *N*-acylpyridinium intermediate during hydrolysis of *p*-nitrophenyl alkanoates **2** catalyzed by **1** in aqueous Tris buffer solution. The **1**-catalyzed hydrolysis reaction of **2** ($n = 2-10$) releases a stoichiometric "burst" of *p*-nitrophenoxide under saturating conditions and depends strongly on the alkyl chain length in the substrate esters. Both acylation and deacylation rates were found to exhibit a maximum for the same substrate **2** ($n = 6$).

Experimental Section

Materials and Reagents. Synthesis of the polysiloxane−bis(trimethylene)-supported 4-(diallylamino)pyridine **1** has been described previously.[9] *p*-Nitrophenyl alkanoates **2** ($n = 2-10$) were purchased from Sigma Chemical Co. Tris(hydroxymethyl)aminomethane and hydrochloric acid were used as received from Fisher.

Kinetic Experiments. The sample cuvette was filled with 2.5 mL of a fresh solution containing catalyst in aqueous Tris buffer solution (0.05 M, pH 8.0), and the solution was equilibrated for 10 min at 30 °C in the thermostated cell compartment of a Hewlett-Packard Model 8450 spectrophotometer. The concentrations of catalyst are expressed in unit mol L^{-1}, where unit molar mass is defined by the molar mass of the repeat unit of copolymer **1**. A stock solution of

[⊗] Abstract published in *Advance ACS Abstracts,* December 15, 1996.

S0024-9297(96)01070-4 CCC: $12.00

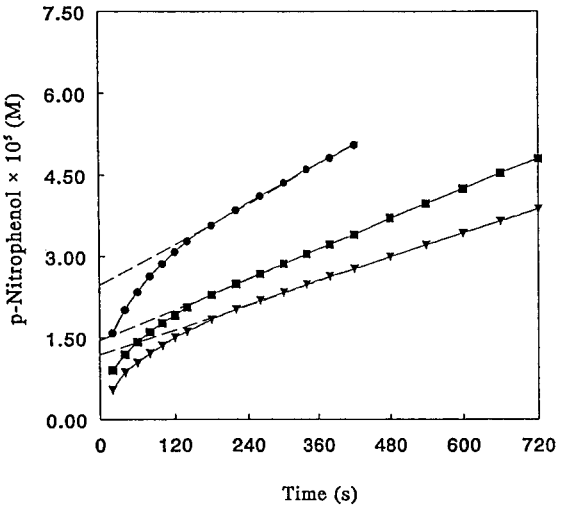


Figure 1. Liberation of *p*-nitrophenoxide in the hydrolysis of *p*-nitrophenyl alkanoates **2** (5.0×10^{-5} M) catalyzed by **1** (5.0×10^{-5} unit mol L^{-1}) for different substrates as a function of time in 0.05 M aqueous Tris buffer solution at pH 8.0 and 30 °C: (●) **2** ($n = 6$); (■) **2** ($n = 4$); (▼) **2** ($n = 2$).

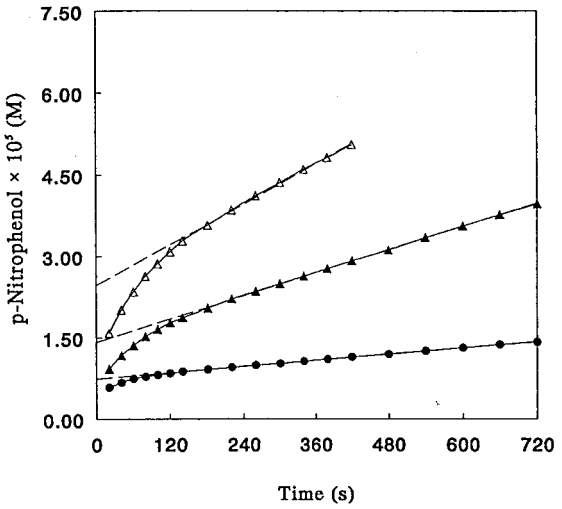


Figure 2. Liberation of *p*-nitrophenoxide in the hydrolysis of **2** (5.0×10^{-5} M) catalyzed by **1** (5.0×10^{-5} unit mol L^{-1}) as a function of time and alkyl chain length in substrate in 0.05 M aqueous Tris buffer solution at pH 8.0 and 30 °C: (△) **2** ($n = 6$); (▲) **2** ($n = 8$); (●) **2** ($n = 10$).

p-nitrophenyl alkanoate **2** ($n = 2-10$) in dioxane (2.5×10^{-2} M) was added by microsyringe. The reaction mixture was quickly mixed by shaking, and the absorbance at 400 nm was recorded as a function of time. Buffer-catalyzed reactions with no added catalyst were performed analogously at the same conditions.

Results and Discussion

We have systematically examined by kinetic analysis the hydrolysis of a series of *p*-nitrophenyl alkanoates **2** ($n = 2-10$) catalyzed by **1** in aqueous solution in order to gain a better understanding of the complicated catalytic process. The hydrolysis reactions were carried out in 0.05 M aqueous Tris buffer solution at pH 8.0 and 30 °C. Figure 1 shows the appearance of the *p*-nitrophenoxide ion in the hydrolysis of **2** ($n = 2$, 4, and 6) catalyzed by **1** as a function of time. The plots of absorbance vs time were corrected to obtain rates of catalyzed reaction by subtracting the concentration of *p*-nitrophenoxide obtained in aqueous Tris buffer solution alone (<5% in most cases studied) and the linear portion of the plot was extrapolated back to zero time. The catalytic hydrolysis reactions for the three substrate esters in Figure 1 (**2**, $n = 2$, 4, and 6) were found to proceed according to typical "burst" kinetics.[17-22] The initial rapid release of *p*-nitrophenoxide is followed by slower, steady release in which the concentration of *p*-nitrophenoxide increases linearly with time. The presence of a steady-state linear portion indicates that the acylation rates are much faster than the deacylation rates in the proposed reaction sequence in Scheme 1. These results suggest that the liberation of *p*-nitrophenoxide is apparently accompanied by the simultaneous formation of an *N*-acylpyridinium intermediate **3**, and the subsequent hydrolysis of this intermediate is rate-limiting in the overall hydrolysis of **2**.[17,21] The liberation of *p*-nitrophenoxide in **1**-catalyzed hydrolysis for **2** ($n =$ 8 and 10) as a function of time is presented in Figure 2. For comparison, the substrate ester **2** ($n = 6$) is also included in Figure 2. Again "burst" kinetics are observed for the substrate esters with longer alkyl chain lengths as evidenced by an initial rapid increase in *p*-nitrophenoxide concentration due to the release of an equivalent of *p*-nitrophenoxide that accompanies formation of **3**. Then, a much slower, turnover stage of the reaction ensues in which excess substrate is hydrolyzed via a sequence of slow hydrolysis of **3** followed by rapid acylation of **1**. Furthermore, we find that the **1**-catalyzed hydrolysis reaction depends strongly on the alkyl chain length in the substrate esters (Figures 1 and 2). The acylation rate increases markedly with increasing alkyl chain length in the substrate esters and reaches a maximum for **2** ($n = 6$). Further increase of the alkyl chain length leads to a decrease in the acylation rate. The deacylation rate was also found to exhibit a maximum for the substrate **2** ($n = 6$). Reduced acylation and deacylation rates for **1**-catalyzed hydrolysis of **2** ($n \geq 8$) may be attributed to formation of homoaggregates and/or heteroaggregates of the substrates and intermediate **3** which bury the substrate ester and **3** in hydrophobic aggregates and isolate these reactive species from the aqueous media. This is consistent with previous observations that *p*-nitrophenyl esters with alkanoate chain lengths greater than six carbons in aqueous media form self-aggregates that cause a retardation of the rate of hydrolysis of **2** ($n \geq 8$) in water.[23-28] Significantly, for the same hydrolysis reaction, the enzyme cholesterol esterase also shows a maximum acylation rate for the same substrate **2** ($n = 6$). The deacylation rate maximizes for **2** ($n = 6$) but drops off precipitously for the substrates **2** ($n = 10$ and 12).[29,30] This indicates that similar structure−activity effects may be operative for the natural and the synthetic catalysts in the hydrolysis process.

The liberation of *p*-nitrophenoxide in **1**-catalyzed hydrolysis of **2** ($n = 4$, 6, and 8) as a function of time with different concentrations of **1** in aqueous Tris buffer solution is shown in Figures 3, 4, and 5, respectively. The results clearly indicate that the magnitude of the "burst" of *p*-nitrophenoxide is proportional with catalyst concentration. In the absence of catalyst, the hydrolysis of **2** ($n = 6$) catalyzed by aqueous Tris buffer solution shows no burst of *p*-nitrophenoxide, and the reaction proceeds at a rate much less than the steady-state stage of catalyzed reactions (Figure 4). These data support a mechanism involving a covalent *N*-acylpyridinium intermediate where the breakdown of the intermediate is the rate-determining step in the **1**-catalyzed hydrolysis reaction (Scheme 1).

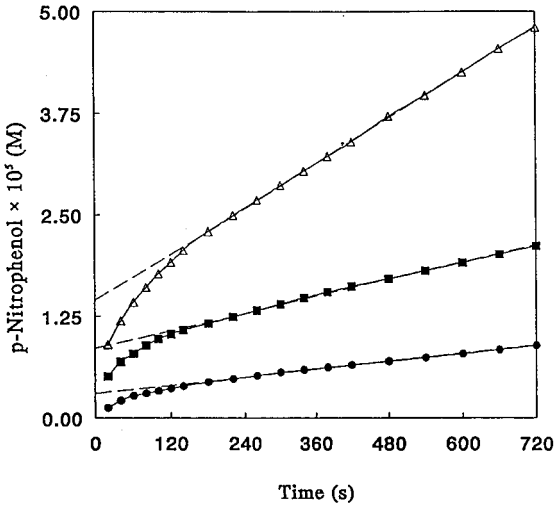


Figure 3. Dependence of the liberation of *p*-nitrophenoxide in the hydrolysis of **2** ($n = 4$, 5.0×10^{-5} M) catalyzed by **1** on catalyst concentration as a function of time in 0.05 M aqueous Tris buffer solution at pH 8.0 and 30 °C: (△) [**1**] = 5.0×10^{-5} unit mol L^{-1}; (■) [**1**] = 2.5×10^{-5} unit mol L^{-1}; (●) [**1**] = 1.0×10^{-5} unit mol L^{-1}.

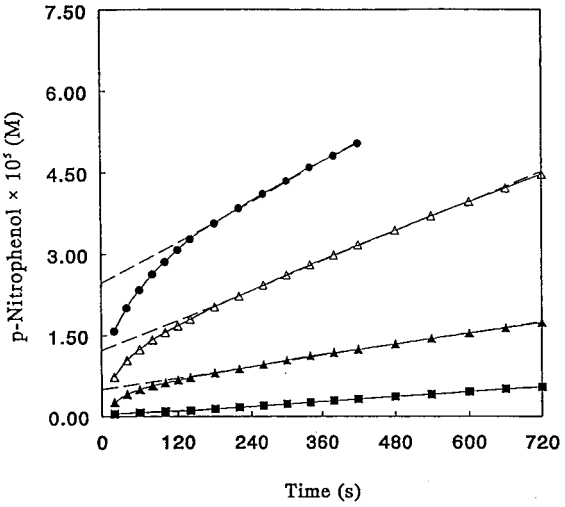


Figure 4. Dependence of the liberation of *p*-nitrophenoxide in the hydrolysis of **2** ($n = 6$, 5.0×10^{-5} M) catalyzed by **1** on catalyst concentration as a function of time in 0.05 M aqueous Tris buffer solution at pH 8.0 and 30 °C: (●) [**1**] = 5.0×10^{-5} unit mol L^{-1}; (△) [**1**] = 2.5×10^{-5} unit mol L^{-1}; (▲) [**1**] = 1.0×10^{-5} unit mol L^{-1}; (■) no catalyst.

Further support for the formation of an *N*-acylpyridinium intermediate **3** is provided by a study of the effect of substrate concentration on the release of *p*-nitrophenoxide for **1**-catalyzed hydrolysis of **2** ($n = 4-8$). The kinetic parameters for these reactions are summarized in Table 1. At low concentrations of substrate, the magnitude of the "burst" of *p*-nitrophenoxide appears to be proportional to the substrate concentration. However, the magnitude of the *p*-nitrophenoxide "burst" is independent of substrate concentration at higher concentrations and equal in concentration to that of catalyst. The reaction shows saturation kinetics in accordance with the Michaelis−Menten model for enzyme reaction kinetics. These results are entirely consistent with a mechanism that requires a covalent *N*-acylpyridinium intermediate **3** and rate-limiting deacylation of the intermediate in the **1**-catalyzed hydrolysis reaction. Under conditions where the concentration of **2** is greater than that of **1**, the catalyst should become saturated with respect to substrate.

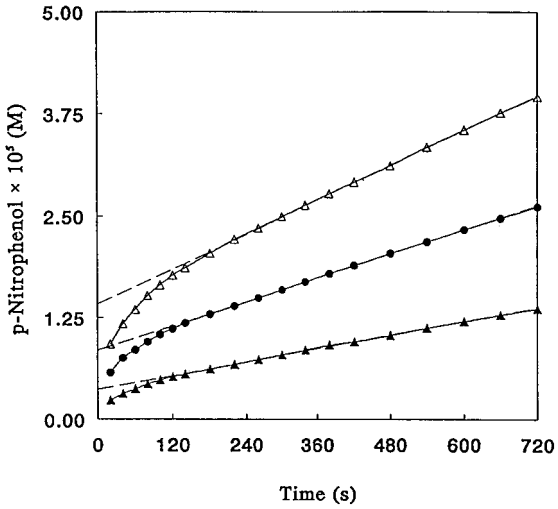


Figure 5. Dependence of the liberation of *p*-nitrophenoxide in the hydrolysis of **2** ($n = 8$, 5.0×10^{-5} M) catalyzed by **1** on catalyst concentration as a function of time in 0.05 M aqueous Tris buffer solution at pH 8.0 and 30 °C: (△) [**1**] = 5.0×10^{-5} unit mol L^{-1}; (●) [**1**] = 2.5×10^{-5} unit mol L^{-1}; (▲) [**1**] = 1.0×10^{-5} unit mol L^{-1}.

Table 1. Kinetic Parameters for 1-Catalyzed Hydrolysis of 2 ($n = 4$, 6, and 8) in 0.05 M Tris Buffer Solution at pH 8.0 and 30 °C[a]

2[b]	$k_a \times 10^2$ (s^{-1})[c]	$k_d \times 10^3$ (s^{-1})[d]	$K_m \times 10^5$ (M)[c]	k_d/K_m (M^{-1} s^{-1})	$V_{max} \times 10^8$ (M s^{-1})[f]
$n = 4$	1.81	3.58	13.6	26.3	3.58
$n = 6$	2.19	4.22	8.29	50.9	4.22
$n = 8$	1.93	2.13	4.45	47.9	2.13

[a] **1**, 1.0×10^{-5} unit mol L^{-1}. [b] The concentrations varied from 0.25 to 1.0×10^{-4} M. [c] k_a, first-order rate constant of the formation of the *N*-acylpyridinium intermediate **3** derived from **1**, which was obtained from the equation $1/k_b = 1/k_a + K_m/(k_a S_0)$ for the presteady state as suggested by Bender *et al.*,[21,22] where k_b is the observed first-order rate constant in the presteady state at $S >$ [cat]. [d] k_d, rate constant of the deacylation of **1**-catalyzed hydrolysis of **2**, which was obtained from the equation $1/V = 1/(k_d[\text{cat.}]_0) + K_m/(k_d[\text{cat.}]_0 S_0)$ for the steady state, where V is the reaction velocity,[21,22] K_m, the Michaelis constant, and V_{max}, the maximum reaction velocity in the Michaelis−Menten model.

Conclusions

The hydrolysis of a series of *p*-nitrophenyl alkanoates **2** ($n = 2-10$) catalyzed by **1** was investigated in aqueous Tris buffer solution. Direct evidence for the existence of an *N*-acylpyridinium intermediate **3** in **1**-catalyzed hydrolysis of **2** ($n = 2-10$) was obtained. Both acylation and deacylation rates were found to be dependent on the alkyl chain length in the substrate esters and to exhibit a maximum for the substrate **2** ($n = 6$). The acylation reaction is first order in catalyst concentration and shows saturation kinetics at high substrate concentration in the aqueous Tris buffer solution. The kinetic behavior of our model system is similar to the behavior of cholesterol esterase-*p*-nitrophenyl alkanoate reactions.[29,30]

Acknowledgment. We thank the Office of Naval Research for financial support of this work.

Supporting Information Available: Plots of the effect of substrate concentration on the release of *p*-nitrophenoxide for **1**-catalyzed hydrolysis of **2** ($n = 4-8$) in aqueous Tris buffer solution (3 pages). Ordering information is given on any current masthead page.

References and Notes

(1) Scriven, E. F. V. *Chem. Soc. Rev.* **1983**, *12*, 129.
(2) Hofle, G.; Steglich, W.; Vorbruggen, H. *Angew. Chem., Int. Ed. Engl.* **1978**, *17*, 569.
(3) Delaney, E. J.; Wood, L. E.; Klotz, I. M. *J. Am. Chem. Soc.* **1982**, *104*, 799.
(4) Hirl, M. A.; Gamson, E. P.; Klotz, I. M. *J. Am. Chem. Soc.* **1979**, *101*, 6020.
(5) Cei, G.; Mathias, L. J. *Macromolecules* **1990**, *23*, 4127.
(6) Mathias, L. J.; Cei, G. *Macromolecules* **1987**, *20*, 2645.
(7) Vaidya, R.; Mathias, L. J. *J. Am. Chem. Soc.* **1986**, *108*, 5514.
(8) Fife, W. K.; Rubinsztajn, S.; Zeldin, M. *J. Am. Chem. Soc.* **1991**, *113*, 8535.
(9) Rubinsztajn, S.; Zeldin, M.; Fife, W. K. *Macromolecules* **1991**, *24*, 2682.
(10) Rubinsztajn, S.; Zeldin, M.; Fife, W. K. *Macromolecules* **1990**, *23*, 4026.
(11) Wang, G. J.; Fife, W. K. *Polym. Prepr. (Am. Chem. Soc., Div. Polym. Chem.)* **1995**, *36* (2), 213.
(12) Fife, W. K. *Trends Polym. Sci.* **1995**, *3*, 214.
(13) Katritzky, A. R.; Duell, B. L.; Seiders, R. P.; Durst, H. D. *Langmuir* **1987**, *3*, 976.
(14) Katritzky, A. R.; Duell, B. L.; Knier, B. L.; Durst, H. D. *Langmuir* **1988**, *4*, 192.
(15) Bhattacharya, S.; Snehalatha, K. *Langmuir* **1995**, *11*, 4653.
(16) Liu, S.; Fife, W. K. *Macromolecules* **1996**, *29*, 3334.
(17) Gitler, C.; Ochoa-Solano, A. *J. Am. Chem. Soc.* **1968**, *90*, 5004.
(18) Ochoa-Solano, A.; Romero, G.; Gitler, C. *Science* **1967**, *156*, 1243.
(19) Kunitaki, T.; Okahata, Y. *J. Am. Chem. Soc.* **1976**, *98*, 7793.
(20) Kunitaki, T.; Okahata, Y.; Sakamoto, T. *J. Am. Chem. Soc.* **1976**, *98*, 7799.
(21) Kezdy, F. J.; Bender, M. L. *Biochemistry* **1962**, *1*, 1097.
(22) Bender, M. L.; Marshall, T. H. *J. Am. Chem. Soc.* **1968**, *90*, 201.
(23) Menger, F. M.; Portnoy, C. E. *J. Am. Chem. Soc.* **1967**, *89*, 4698.
(24) Menger, F. M.; Portnoy, C. E. *J. Am. Chem. Soc.* **1968**, *90*, 1875.
(25) Guthrie, J. P. *Can. J. Chem.* **1973**, *51*, 3494.
(26) Blyth, C. A.; Knowles, J. R. *J. Am. Chem. Soc.* **1971**, *93*, 3021.
(27) Jiang, X.-K. *Acc. Chem. Res.* **1988**, *21*, 362.
(28) Jiang, X.-K.; Ji, G.-Z.; Tu, B.; Zhang, X.-Y.; Shi, J.-L.; Chen, X. *J. Am. Chem. Soc.* **1995**, *117*, 12679.
(29) Sutton, L. D.; Stout, J. S.; Quinn, D. M. *J. Am. Chem. Soc.* **1990**, *112*, 8398.
(30) Sutton, L. D.; Quinn, D. M. *J. Am. Chem. Soc.* **1990**, *112*, 8404.

MA961070M