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Phosphate Ester Cleavage Catalyzed by Bifunctional Zinc Complexes: Comments on the "*p*-Nitrophenyl Ester Syndrome"

RONALD BRESLOW[1] AND SHARAT SINGH

Department of Chemistry, Columbia University, New York, New York 10027

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A tetra-aza macrocyclic Zn^{2+} complex has been attached to β-cyclodextrin. The resulting compound is a better catalyst for the hydrolysis of *p*-nitrophenyl diphenyl phosphate than is the simple complex and shows Michaelis-Menten binding kinetics. The zinc macrocyclic complex has also been dimerized by a covalent *p*-phenylene linkage. The dimer is an improved hydrolytic catalyst, both for the phosphate ester and for *p*-nitrophenyl acetate. The use and misuse of *p*-nitrophenyl esters in enzyme model systems are briefly discussed.

INTRODUCTION

We have described the hydrolysis of *p*-nitrophenyl diphenyl phosphate (**1**) catalyzed by the zinc macrocyclic complex **2** (*1*). The macrocyclic ligand in **2** is so far almost unique in binding Zn^{2+} so strongly that a bound zinc hydroxide species can be formed in base without extraction of the zinc from the ligand. From our evidence, the zinc was acting as a bifunctional catalytic center; it furnished a nucleophilic OH^- group while at the same time stabilizing the developing O^- of the phosphate by Lewis acid coordination. Even more effective was hydrolysis of **1** in a micelle containing catalyst **3**, whose long alkyl chain caused it to insert and orient in the micelle (*1*). The micellar phase binds both the substrate **1** and the catalyst **3** into the same microenvironment (*2*), but not in well-defined proximity. Thus we decided to construct a new catalyst **4** carrying a cyclodextrin group to bind the substrate next to the zinc.

Our kinetic studies (*1*) on catalyst **3** revealed a rate term second-order in catalyst, suggesting hydrolysis of **1** by two cooperating zinc complexes in the micelle. This might well involve the two classic modes of metal catalysis: one zinc acting as a Lewis acid to stabilize the developing O^- of the phosphate group, while a different one acted to furnish bound hydroxide as a nucleophile. For this reason we have constructed the dimeric catalyst **5**, with a spacer holding the two rings apart to prevent μ-oxo metal bridging. Such bridging would correspond to a "short circuit," by which the acidic and basic groups directly interact. In **5**,

[1] To whom all correspondence should be addressed.

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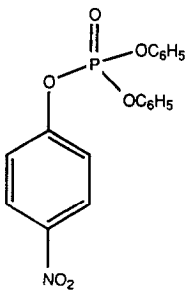

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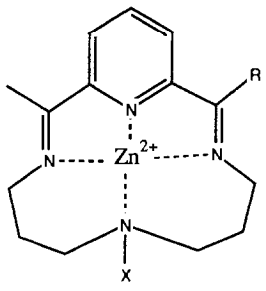
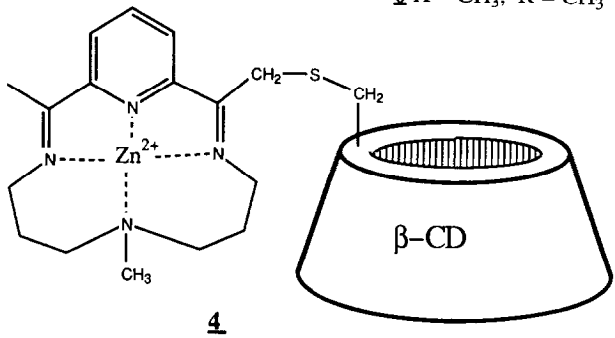


2 X = H, R = CH_3
3 X = CH_3, R = n-$C_{17}H_{35}$
6 X = CH_3, R = CH_3



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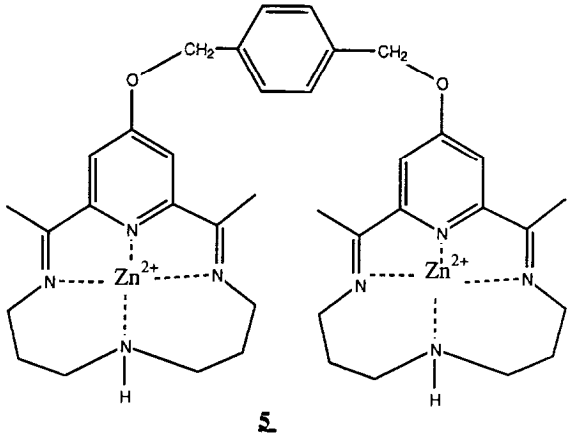

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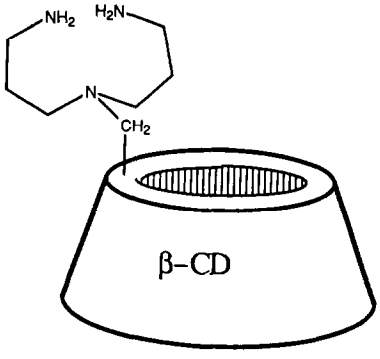
models show that direct interaction of both zincs with a single hydroxide ion is not possible; the electron flow can however occur through an intervening phosphate group, as needed for catalysis. Catalysts **4** and **5** have both proved to be more effective at cleaving **1** than is the simple catalyst **2.**

For the attachment of a β-cyclodextrin unit to complex **2** or to its *N*-methyl derivative **6,** we considered several possible approaches. In one we attached dipropylenetriamine to C-6 of β-cyclodextrin to form **7** and then tried to construct the macrocycle (*3*) by reaction of **7** with diacetylpyridine and Zn^{2+}. In our hands this approach failed, although we were able to prepare the corresponding Cu^{2+}



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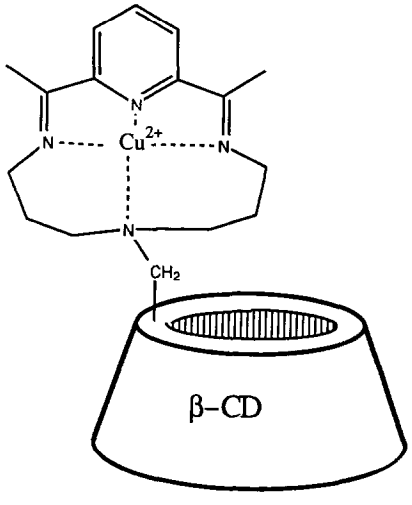
complex **8** in this way. Thus we set out to prepare the thiol derivative **9** of macrocyclic complex **6.**

It seemed likely that the methyl groups of **6** are acidic, and this proved to be the case. With NaH an anion was easily formed, which we used to attach a thiol group. Although various disulfides have been used to convert carbanions to protected thiols (*4, 5*), we found that reaction of the anion of **6** with S_8 (*6*) produced the thiol **9** conveniently. With β-cyclodextrin-6-iodide this yielded the target compound **4.**

For the synthesis of **5** we converted 2,6-diacetylpyridine to its bis-ethylene ketal **10.** This was oxidized to the *N*-oxide **11,** which with acetic anhydride afforded the 4-acetoxypyridine derivative **12.** Hydrolysis during workup yielded the diacetyl-γ-pyridone diketal **13,** which was *O*-alkylated with *p*-xylylene dibromide to afford the diether **15.** Then acid hydrolysis afforded the tetraketone **16,** and with dipropylenetriamine and $ZnBr_2$ this was converted to **5.**



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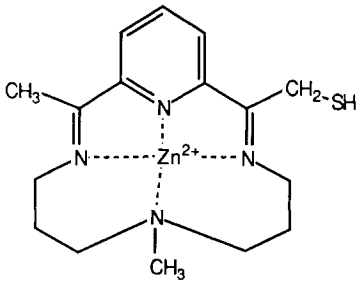

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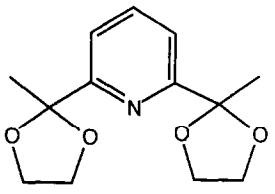

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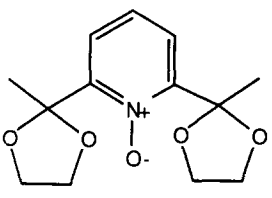

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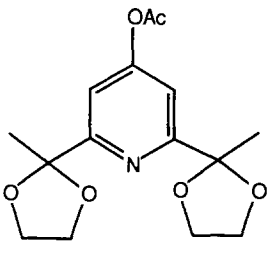

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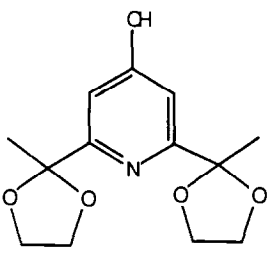

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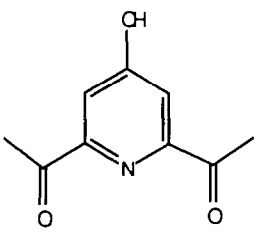

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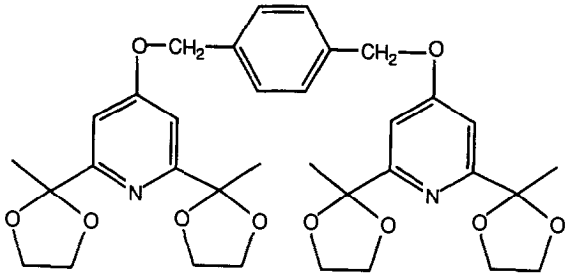
EXPERIMENTAL

Synthesis of compounds. Complex **6** (*3*) (517 mg) was treated with 29 mg (1.2 eq) of NaH in 5 ml DMF[2] and 5 ml tetrahydrofuran under argon at −78°C for 2 h and then with 1 eq S_8 over 12 h while warming to room temperature. After aqueous workup the resulting crude **9** was directly treated with an excess (4 eq) of β-cyclodextrin-6-iodide in 5 ml of DMF with a few eq of 5% K_2CO_3 in methanol at 60°C for 3 days (argon), to afford **4** in 12% yield after purification by ion exchange (Cm-25, 1 M ammonium carbonate) and gel (Bio-Rad P4, 0.1 M ammonium carbon-

[2] Abbreviations used: DMF, dimethylformamide; CI-MS, chemical ionization mass spectrometry; CD, cyclodextrin.



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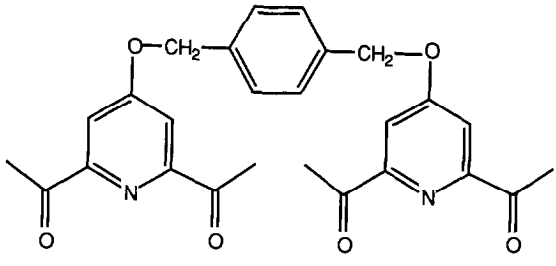

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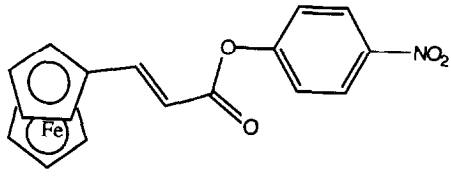

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ate) chromatography. The compound had the expected ^{1}H NMR spectrum, with a 3 : 7 ratio of pyridine protons to anomeric C-1 protons, and the fast atom bombardment mass spectrum (with NaCl) showed peaks at 1521 (**4**-Cl)$^+$ and 1503 (**4**-OH)$^+$.

2,6-Diacetylpyridine (1.63 g) with 25 ml ethylene glycol was added to 100 ml dry benzene. Then 10 ml trimethylsilyl chloride was added, and the solution was heated under reflux for 24 h with water separation. Washing with 5% K_2CO_3, then H_2O, and solvent evaporation yielded 2.43 g (100% yield) of crystalline **10**, mp 64–67°C. In the ^{1}H NMR **10** showed signals at δ 1.8 (s, 6H), 4.0 (m, 8H), 7.5 (d, 2H), and 7.7 (t, 1H).

A solution of **10** (2.43 g) and *m*-chloroperbenzoic acid (4.68 g, 3 eq) in 100 ml dry methylene chloride was heated under reflux for 6 h. Washing four times with 10% aqueous K_2CO_3 and then H_2O, drying (K_2CO_3), and solvent evaporation gave a

crude product that could be separated by flash chromatography (*7*) (silica, EtOAc) to yield 1.3 g of recovered **10** and 1.1 g (91% yield based on recovered **10**) of **11.** In the ^{1}H NMR **11** had signals at δ 2.0 (s, 6H), 4.0 (m, 8H), 7.15 (t, 1H), and 7.6 (d, 2H), indicating increased shielding of the γ hydrogen.

Heating **11** (6 g) in 150 ml acetic anhydride at 70°C for 24 h rearranged it. The reaction mixture was added to crushed ice and the products were isolated by ether extraction. After 5% K_2CO_3 washing and Na_2SO_4 drying, chromatography afforded 4.97 g of a mixture of **12** and **13,** along with a small amount of the diketone **14** from hydrolysis of **13.** The compounds could be separated by flash chromatography (silica, EtOAc) to yield **13** (52%) as a yellow viscous oil with ^{1}H NMR signals at δ 1.8 (s, 6H), 4.0 (m, 8H), and 7.55 (s, 2H).

A solution of **13** (520 mg) with *p*-xylylene dibromide (276 mg) and Ag_2CO_3 (864 mg) was heated to reflux in 20 ml of dry benzene under N_2 for 24 h with the exclusion of light. Isolation by flash chromatography afforded **15** (39% yield) as a yellow liquid, with CI-MS (CH_4) 637. After 2 h at reflux with 1 N HCl in dioxane, **15** was converted quantitatively to **16,** which had CI-MS (CH_4) 461 and the expected NMR signals at δ 2.8, 4.5, 7.3, and 8.2.

The bis-complex **5** was prepared by heating **16** (460 mg, 1 eq) with $ZnBr_2$ (225 mg, 2.1 eq) and dipropylenetriamine (131 mg, 2.2 eq) in aqueous ethanol at reflux for 16 h, under the standard conditions (*3*) (ca. 0.2 M concentrations) for the preparation of such complexes. After solvent evaporation the compound was isolated as the mixed dibromide diperchlorate salt (by adding saturated aqueous $NaClO_4$) in 29% yield. Dimer **5** showed the characteristic ^{1}H NMR signals near 3 and 4 ppm for the propylene units, a sharp singlet at 2.6 ppm for the CH_3 groups, and phenyl and pyridine protons, all with the correct integration. In impure samples a small signal at 2.8 ppm indicated some residual acetyl groups.

Titration of 5. A solution of **5** (2.3 mg) in 2 ml of 50% H_2O/CH_3CN was titrated at 25.0°C against a glass electrode with 0.25 M NaOH in H_2O. The resulting curve (Fig. 1) shows no obvious break, with 2.6 eq (expected 2.0) of OH^- consumed

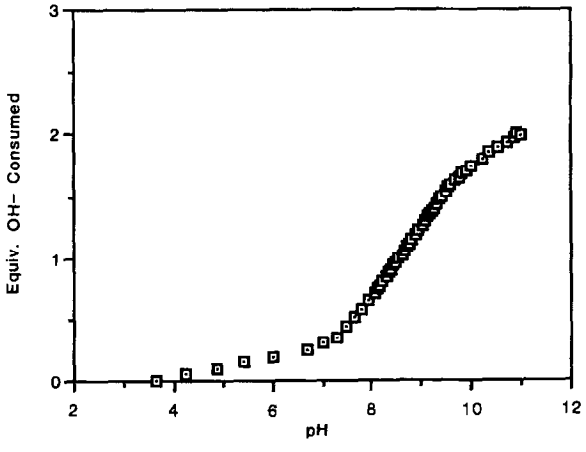

FIG. 1. Titration of bis-complex **5** with NaOH at 25.0°C. The base consumed was corrected for the titration curve of solvent alone in this pH region.

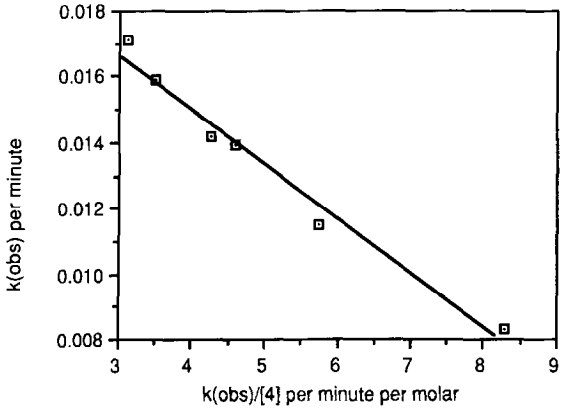

FIG. 2. An Eadie plot of the observed catalytic hydrolysis rates at 25.0°C for the cleavage of phosphate **1** by various concentrations of CD-complex catalyst **4**, corrected for the slow hydrolysis in the absence of catalyst.

over the pH region 7.9 to 10.0 (the curve was corrected by titration of solvent alone over the same pH range). The curve is, however, broader than a titration curve of **2** under the same conditions.

Kinetic methods. The rate of hydrolysis of **1** was followed spectrophotometrically (*1*) by observing the appearance of *p*-nitrophenoxide ion at 400 nm using a Beckman DUB-8 thermostated at 25.0°C. The reaction medium was 0.9 ml of 10 mM aqueous phosphate buffer, pH 8.00, and 0.3 ml of acetonitrile. The reaction was initiated by addition of 10 μl of a stock solution of **1** in acetonitrile, making a final concentration of 0.100 mM in **1.** Catalysts were at 1 mM except in the experiment with **4** in which 1 mM to 5.5 mM concentrations were used to derive an Eadie plot. The reactions were followed to at least 90% conversion. The reported pseudo-first-order rate constants are an average of at least three runs, which agreed within 20%.

Hydrolysis of **1** by catalyst **5** was studied with pH 8 Epps[3] buffer in 70% aqueous MeCN, as described previously (*1*). This buffer could not be used with **4,** because it binds to the cyclodextrin group. For hydrolysis by **4** an Eadie plot (*8*) of k_{obs} vs $k_{obs}/[\mathbf{4}]$ was used to derive K_{diss} and k_{cat}. Six different concentrations were used; the data are plotted in Fig. 2.

RESULTS AND DISCUSSION

Titration of the dimeric metallocycle **5** (H_2O/MeCN) showed a two OH^- reaction from pH 7.9 to 10 without a discernible break (Fig. 1), indicating that the first OH^- cannot bind to both Zn's. Such μ bridging would have led to stronger binding

[3] Obtained from Sigma Chemical Co. and used without further purification. This buffer (*N*-[2-hydroxyethyl]piperazine-*N*'-3-propanesulfonic acid) does not bind well to metals, but it does bind into the cyclodextrin cavity, so it was not used with **4.**

TABLE 1

Second-Order Catalytic Rate Constants (25.0°C)

Catalyst	Substrate	$k_2 \times 10^2$ (M^{-1} s^{-1})
Simple complex **2**	Phosphate **1**	3.80 ± 0.05
Methyl complex **6**	Phosphate **1**	3.05
bis-complex **5**	Phosphate **1**	16.91 ± 0.08
CD-complex **4**	Phosphate **1**	21.7[a]
Simple complex **2**	Acetate[b]	5.03 ± 0.03
bis-complex **5**	Acetate[b]	35.00 ± 0.05

[a] k_{cat}/K_{diss} ($\times 10^2$) where k_{cat} is 3.63×10^{-4} s^{-1} and K_{diss} is 1.67×10^{-3} M.

[b] *p*-Nitrophenyl acetate.

of the first OH^-, weaker binding of the second OH^-, and a break in the titration curve.

The kinetic data from these studies are listed in Table 1. As that table shows, the dimer **5** proved to be 4.4 times as effective as is the monomer **2** in the hydrolysis of **1.** We have also examined it in the hydrolysis of *p*-nitrophenyl acetate, in which **5** proved to be seven times as effective as is the monomer **2.** Thus the dimerization of macrocycle **2** in structure **5** does indeed result in improved catalysis, but the improvement is not large. Presumably more rigid structures than **5** would be more effective. However, we showed (*1*) that even with monomer **2** it was likely that bifunctional catalysts was occurring in the hydrolysis of **1,** although not in the hydrolysis of *p*-nitrophenyl acetate. A dimer such as **5** has the potential only to substitute a different type of bifunctional catalysis in the hydrolysis of **1,** centered on two metals instead of one.

The binding catalyst **4** shows complex kinetics in the hydrolysis of phosphate ester **1,** with K_{diss} of 1.7 mM and k_{cat} of 3.6×10^{-4} s^{-1} in 80% 10 mM aqueous phosphate buffer (pH 8), 20% MeCN. The K_{diss} is of the general magnitude expected (*9*) for binding of a *p*-nitrophenyl group into a β-cyclodextrin (cf. Fig. 3). The k_{cat}/K_{diss} for reaction of **4** with **1** is seven times as great as is k_{cat} for the unbinding analog **6.** This is the correct comparison in relating a binding to a nonbinding catalyst: k_{cat}/K_{diss} is a second-order rate constant corresponding to reaction without kinetic saturation (*10*). Thus the binding group in **4** adds a real improvement, but by only a factor of 7. Presumably this modest but real improvement of the already good catalyst **6** would also be even greater with more rigid linkages between the segments of the catalyst.

Since the substrate (**1**) in these studies is a *p*-nitrophenyl phosphate ester, we must address the question of the significance of this choice. In a recent communication Menger and Ladika discussed what the authors called the "*p*-nitrophenyl ester syndrome" (*11*). They pointed out that the catalytic cleavage of reactive *p*-nitrophenyl esters by various enzyme models is by no means equivalent to the enzymatic cleavage of peptides or even of normal esters. This point has been

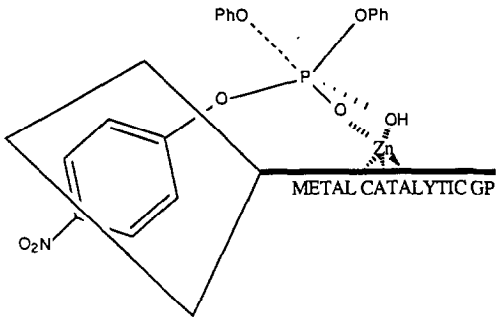

FIG. 3. The transition state for cleavage of **1** by catalyst **4**. Molecular models show that this geometry is indeed possible.

made before (*12, 13*). They also extended our previously described reaction (*12, 14*) of β-cyclodextrin with ferroceneacrylate derivates and reported that the very large acceleration we had seen with the *p*-nitrophenyl ester (**17**) was not seen with esters carrying poorer leaving groups. They suggested that the problem was a change in rate-determining step, from *formation* of the tetrahedral intermediate (the first step) with a good leaving group such as *p*-nitrophenoxy to *loss* of the leaving group and formation of the product ester (the second step) with less active esters. They posited that the product was destabilized because it had the s-*cis*-ester geometry, thus slowing the second step.

Although they did not mention it, we had already called attention to this leaving group effect in one of our major papers (*15–17*) and had pointed out that the second step should become rate-determining with poorer leaving groups, as we observed (*15–17*). We had offered a different geometric explanation of the problems with this second step: twisting of the conjugated system in the product. Molecular models and computer modeling can be used to distinguish between these possible explanations of the phenomenon that we had first called attention to, and that is clearly supported by the data of Menger and Ladika: the large accelerations seen with *p*-nitrophenyl esters are *not* seen with poorer leaving groups.

The use of a *p*-nitrophenoxy leaving group in **17** allowed us to study the huge accelerations of the first step in acylation ($>10^5$) produced by the geometry of the complex of **17** with β-cyclodextrin and the even larger accelerations with analogs of **17** (*12*). These very large accelerations of the first step, and the interesting geometric problems that occur during the second step, would have been hidden with a poorer leaving group. In our current work, the *p*-nitrophenoxy group in substrate **1** is analogous to the good leaving groups in nerve gases; thus studies aimed at the detoxification of nerve gases have often used this substrate (*2*).

The study of *p*-nitrophenyl ester reactions represents a "syndrome" only if fast cleavage of nitrophenyl carboxylic esters is misrepresented as solving the problem of mimicking peptidases. Absent such misrepresentation, *p*-nitrophenyl esters should continue to play an important role in the development and understanding of artificial enzymes.

ACKNOWLEDGMENTS

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REFERENCES

1. GELLMAN, S., PETTER, R., AND BRESLOW, R. (1986) *J. Amer. Chem. Soc.* **108,** 2388.
2. MOSS, R. A., ALWIS, K. W., AND SHIN, J. S. (1984) *J. Amer. Chem. Soc.* **106,** 2651; MENGER, F. M., GAN, L. H., JOHNSON, E., AND DURST, D. H. (1987) *J. Amer. Chem. Soc.* **109,** 2800.
3. KAHN, J. L., AND BUSCH, D. H. (1966) *Nature* **211,** 160.
4. TROST, B. M., AND SALZMANN, T. N. (1973) *J. Amer. Chem. Soc.* **95,** 6840.
5. SEEBACH, D., AND TESCHNES, M. (1973) *Tetrahedron Lett.*, 5113.
6. SEEBACH, D., AND TESCHNES, M. (1976) *Chem. Ber.* **109,** 1601.
7. STILL, W. C., KAHN, M., AND MITRA, A. (1978) *J. Org. Chem.* **43,** 2923.
8. EADIE, G. S. (1942) *J. Biol. Chem.* **146,** 85.
9. BENDER, M. L., AND KOMIYAMA, M. (1978) Cyclodextrin Chemistry, Springer-Verlag, New York.
10. WALSH, C. (1979) Enzymatic Reaction Mechanisms, p. 34, Freeman, San Francisco.
11. MENGER, F. M., AND LADIKA, M. (1987) *J. Amer. Chem. Soc.* **109,** 3145.
12. BRESLOW, R., TRAINOR, G., AND UENO, A. (1983) *J. Amer. Chem. Soc.* **105,** 2739.
13. BRESLOW, R. (1986) *in* Chemical Reactions in Organic and Inorganic Constrained Systems (Setton, R., Ed.), pp. 17–28, Reidel, Amsterdam.
14. (a) CZARNIECKI, M. F., AND BRESLOW, R. (1978) *J. Amer. Chem. Soc.* **100,** 7771; (b) BRESLOW, R., CZARNIECKI, M. F., EMERT, J., AND HAMAGUCHI, H. (1980) *J. Amer. Chem. Soc.* **102,** 762; (c) TRAINOR, G. L., AND BRESLOW, R. (1981) *J. Amer. Chem. Soc.* **103,** 154; (d) LE NOBLE, W. J., SRISTAVA, S., BRESLOW, R., AND TRAINOR, G. (1983) *J. Amer. Chem. Soc.* **105,** 2745.
15. BRESLOW, R., TRAINOR, G., AND VENO, A. (1983) *J. Amer. Chem. Soc.* **105,** 2742–2743.
16. BRESLOW, R. (1986) *in* Chemical Reactions in Organic and Inorganic Constrained Systems (Setton, R., Ed.), pp. 18–20, Reidel, Amsterdam.
17. CZARNIECKI, M. F., AND BRESLOW, R. (1978) *J. Amer. Chem. Soc.* **100,** footnote 9.