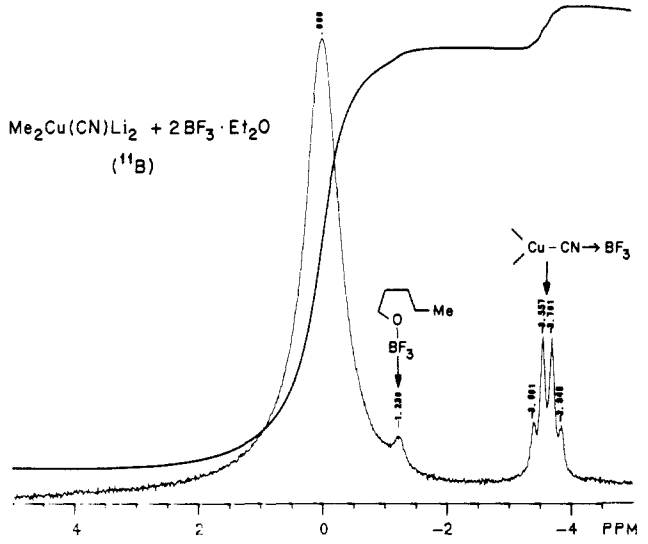


Figure 2. ^{11}B NMR spectrum of $Me_2Cu(CN)Li_2 + 2BF_3{\cdot}Et_2O$ in THF at −70 °C.

two new peaks in the PMR spectrum, in addition to that seen for **6** alone (Figure 1). The new species are identified as the L.O. cuprate MeCu(CN)Li and $MeLi{\cdot}BF_3$.[9] For **7**, which alone shows two methyl singlets suggesting geometrical isomers of a presumed dimer,[10] addition of BF_3 results in essentially complete disappearance of these peaks at −70 °C, with the same two peaks being produced as with **6** but in differing amounts. Cuprate **8** in the presence of BF_3 shows mostly $MeLi{\cdot}BF_3$, plus small amounts of the two signals corresponding to the original cuprate.

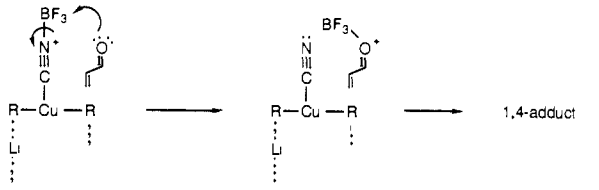
Especially informative data was secured by ^{11}B NMR experiments which shed light not only on the PMR data above but also to the physical location as to some of the BF_3 in the medium. As shown in Figure 2, **6** displays a broad, small signal at δ −1.24, while a stand-alone quartet appears at δ −3.63 (J = 23 Hz). The downfield signal is due to ring opening of THF induced by BF_3, while that at −3.63 ppm *is unequivocally assigned to the Lewis acid on the nitrogen of the nitrile ligand.*[11] With both **7** and **8** the same peaks are present, although with **7** a third signal is observed at −0.54 ppm due to BF_3 complexation of $MeOC(CH_3)_2C{\equiv}C{-}Li$.[8]

These data can be summarized as follows: (1) irrespective of the H.O. cuprate, BF_3 sequesters RLi from the cuprate cluster; (2) with H.O. homocuprates ($R_2Cu(CN)Li_2$), BF_3 rapidly generates an equilibrium as in eq 2; (3) for H.O. mixed cuprates ($RR'Cu(CN)Li_2$), an equilibrium is also established which strongly favors formation of four components, as in eq 3; (4) the BF_3 is

$$R_2Cu(CN)Li_2 + BF_3 \rightleftharpoons RCu(CN)Li + RLi{\cdot}BF_3 \quad (2)$$

$$2RR'Cu(CN)Li_2 + 2BF_3 \rightleftharpoons RCu(CN)Li + R'Li{\cdot}BF_3 + R'Cu(CN)Li + RLi{\cdot}BF_3 \quad (3)$$

situated on the nitrile group as part of the H.O. cuprate.[12] Since the species (in all cases) responsible for the actual chemistry is the H.O. cuprate,[13] a new proposal which accounts for the oftentimes huge increases in reaction rates emerges. That is, rather than as in eq 1, the BF_3 immediately associates (to varying degrees) with the nitrile ligand *within the cuprate* thereby providing strong intra-aggregate activation of the enone, as illustrated below.



In conclusion, with the aid of chemical studies[14] it now seems unlikely that the role of this Lewis acid in accelerating cuprate reactions can be ascribed solely to substrate activation. More definitive information has been gleaned from proton and ^{11}B NMR data on various cuprate/BF_3 solutions. These experiments provide prima facie evidence for cuprate modification *prior to arrival of a substrate* as well as allow for a reasonable alternative explanation of the pronounced effects of $BF_3{\cdot}Et_2O$ on H.O. cuprate reactions.

Acknowledgment. Financial support provided by the donors of the Petroleum Research Fund, administered by the American Chemical Society, and the National Science Foundation (CHE 87-03757) is gratefully acknowledged.

Supplementary Material Available: Proton NMR spectra for $Me_2Cu(CN)Li_2$, $Me(MeOC(CH_3)_2C{\equiv}C)$-$Cu(CN)Li_2$ (**7**), **7** + $2BF_3{\cdot}Et_2O$, $Me(2\text{-Th})Cu(CN)Li_2$ (**8**), and **8** + $2BF_3{\cdot}Et_2O$ and ^{11}B NMR spectra for **7** + $2BF_3{\cdot}Et_2O$ and **8** + $2BF_3{\cdot}Et_2O$ (4 pages). Ordering information is given on any current masthead page.

(9) Control experiments were conducted to confirm the identity of all of the species arising from the original cuprate solutions upon exposure to $BF_3{\cdot}Et_2O$.

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(12) At ca. −75 °C, only a percentage of the nitrile ligand is complexed by BF_3. Upon warming to ca. −55 °C, a significantly greater amount of Lewis acid is irreversibly bound to the CN group, as recooling does not alter the revised (^1H, ^{11}B) NMR spectra.

(13) This seems clear on the basis of (1) comparison data on diastereomer ratios obtained by using the L.O. cyanocuprates plus $BF_3{\cdot}Et_2O$; (2) slightly faster rates of reactions with H.O. versus L.O. cuprates and BF_3 with **1**; (3) ^{11}B NMR spectra of L.O. cyanocuprates and BF_3 which show much less complexation of the Lewis acid with the nitrile ligand.

(14) The role of the crown ether in these reactions is presumably one of enhancing the steric bulk of the reagent via association with the cuprate as the Li^+ adduct. Treatment of $Me_2Cu(CN)Li_2$ with 15-Cr-5 leads to a highly turbid mixture in THF. Addition of BF_3 solubilizes most of the species, the ^{11}B NMR of which is essentially the same as seen with $Me_2Cu(CN)Li_2 + BF_3$. This increase in size would explain the enhanced diastereoselectivity observed.

Antibody Catalysis of Bimolecular Amide Formation†

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The potential for antibodies to accelerate chemical reactions derives from their specific ligand binding function. By designing appropriate substances to be used as haptens, we may elicit antibodies with combining sites that are expedient to enzyme-like activity and specificity.[1] Antibodies to phosphonate esters have been shown to catalyze acyl transfer reactions of carboxylic esters resulting in hydrolysis[2,3] and lactonization.[4]

* Authors to whom correspondence should be addressed.
† Dedicated to Professor E. J. Corey on the occasion of his 60th birthday.
‡ This is Contribution No. 5326-MB from the Department of Molecular Biology.

(1) Jencks, W. P. *Catalysis in Chemistry and Enzymology*; McGraw-Hill: New York, 1969; p 288. Lerner, R. A.; Tramontano, A. *Sci. Am.* **1988**, *253(3)*, 58-70. Lerner, R. A.; Tramontano, A. *Trends Biochem. Sci.* **1987**, *12*, 427. Tramontano, A.; Janda, K.; Napper, A. D.; Benkovic, S. J.; Lerner, R. A. *Cold Spring Harbor Symp. Quant. Biol.* **1987**, *52*, 91. Schultz, P. G. *Science (Washington, D.C.)* **1988**, *240*, 426.

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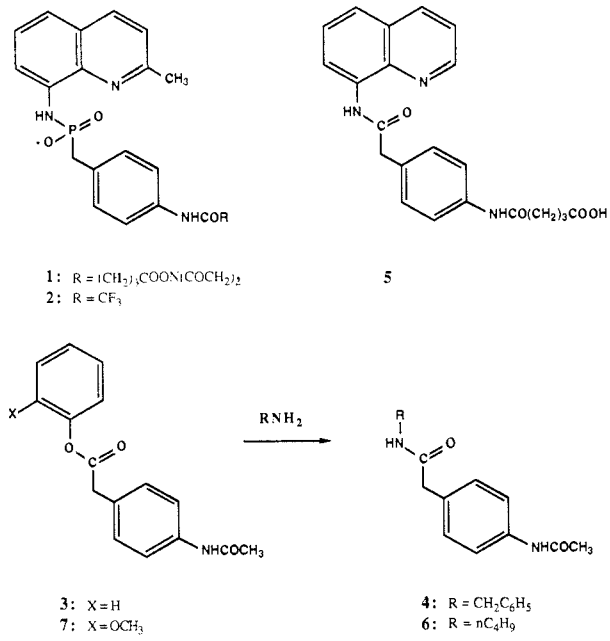
Scheme I



1: R = $(CH_2)_3CONHCOCH_2)_2$
2: R = CF_3

5

3: X = H
7: X = OCH_3

4: R = $CH_2C_6H_5$
6: R = nC_4H_9

We have been exploring the properties of *anti*-phosphonamidate antibodies as catalysts for equivalent acyl transfer processes in which an amine group is the acyl donor (amidolysis) or the acceptor (amide synthesis). Antibodies examined in this study failed to demonstrate hydrolytic activity on aryl amide substrates. However, aryl esters were subject to catalyzed hydrolysis and aminolysis by some of these antibodies. Herein, we describe an *anti*-phosphonamidate antibody active in amide synthesis from ester and amine substrates.

Among a group of 55 monoclonal antibodies (mAb) to the aryl phosphonamidate **1**,[5] 13 were discerned to have measurable activity in hydrolysis of an aryl ester.[6] Though these are relatively poor catalysts in comparison to other esterolytic antibodies,[7] good substrate binding (as estimated by K_M) is observed. The phenyl ester **3** is, in general, only a poor hydrolytic substrate for these antibodies, and a complex of *anti*-**1** mAb with **3** might be expected to bind certain aromatic amines to form a ternary complex capable of acyl transfer to nitrogen. Indeed, one of these antibodies (17G8) augmented the rate of reaction of **3** with benzylamine.

The formation of benzamide **4** was observed by HPLC analysis[8] of a mixture **3** (0.8 mM) and benzylamine (20 mM) in phosphate buffer (50 mM) at pH 8.0 and 23 °C. In the absence of catalyst the ester is mostly hydrolyzed in 35 h, and 40 μM of **4** (5% yield) is formed. In the presence of 17G8 (20 μM), 160 μM of **4** (20%) was obtained under otherwise identical conditions. The amide formation due to antibody represents a turnover ratio of about 4. Other *anti*-**1** mAb's or nonspecific mouse mAb did not increase the rate of formation of **4** under these conditions.

Saturation kinetics were observed with both substrates, as determined by measurement of initial rates.[9] When the antibody is saturated with respect to benzylamine (20 mM), the values of constants $k_{cat} = 0.023$ min^{-1} and K_M(ester) = 2.2×10^{-3} M were estimated from double reciprocal plots of initial rate data. The haptenic ligand **2** inhibited the reaction as expected, with 50% inhibition occurring at about 10 μM.[10] Inhibition of the hydrolytic or aminolytic activity of mAb 17G8 by an aryl amide **5** (0.5 mM), which could be considered a ground-state analogue, was not detected.

The rate of formation of glycyl amide from glycine (10 mM) and **3** (0.8 mM) was not affected by mAb 17G8. Selectivity for aromatic vs aliphatic amines was observed in the competition between benzylamine and *n*-butylamine (10 mM each) reacting with **3** (0.5 mM). The ratio of **4** to **6** due to mAb 17G8 (20 μM) after 4 h is about 3.7 (corrected for background), while in the absence of mAb 17G8 it is 0.72. This specificity was found to depend on the structure of the ester leaving group. The same competition experiment performed with ester **7** (0.5 mM) as substrate produced 6.1 μM of benzyl amide and none of the butyl amide in 4 h. Since the limit of detection in the HPLC assay is about 0.2 μM, the ratio of **4** to **5** is at least 30, whereas in the absence of mAb 17G8 the ratio is about 0.9. The rate of amide formation from the chiral α-methylbenzylamine was slower but seemed to depend on the configuration. (*R*)- and (*S*)-α-methylbenzylamine (20 mM) were reacted separately with **3** (0.5 mM) in the presence of mAb 17G8 (20 μM). The (*S*)-amine produced 1.1 μM of amide in 4 h, while (*R*)-amine produced only 0.5 μM in this time, indicating an *S*:*R* selectivity ratio of about 2.2.

The second-order rate constant for the uncatalyzed formation of **4** at pH 8.0 determined by initial rates was 2.2×10^{-3} M^{-1} min^{-1}. A comparison of this to the first-order constant k_{cat} for the antibody catalyzed reaction reveals an effective molarity[11] of about 10.5 M. The antibody 17G8 is shown to be selective for substrates in accord with the structural characteristics of its hapten **1**. Benzylamine is presumably a better substrate than butylamine or glycine because of its aromatic group, which may be bound at the subsite having affinity for the quinoline group of **1**. The rate differential observed with (*S*)- and (*R*)-α-methylbenzylamine as substrates is evidence for the influence of the chiral environment of an antibody combining site. The mechanism of the reaction may require the antibody binding two substrates in proximity. The intermediacy of an acylated antibody cannot be ruled out, but if one were involved, its partitioning to products would not be expected to depend on the phenol structure.[12] The different ratios of benzyl and butyl amides formed from esters **3** and **7** is evidence against a double displacement mechanism provided that the mechanism remains the same for the two substrates.

While previously described mAb mediated hydrolyses are formally bimolecular, it is difficult to demonstrate the binding of water as a substrate. An earlier attempt to accelerate Schiff's base formation with polyclonal antibodies gave no evidence for the acceleration of a bimolecular reaction.[13] More recently, a communication suggests that antibody can organize two substrates for a photochemical reaction.[14] However, this process has not

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(5) The preparation of phosphonamidate **1** begins with the previously described phosphonyl chloride[2b] and proceeds by the following sequence: (i) addition of lithio-8-aminoquinaldine, THF, −20 °C; (ii) amine deprotection, Na_2CO_3 (10 equiv), 10% aqueous MeOH, 25 °C, 12 h; (iii) acylation, $(CH_2CO)_2NOOC(CH_2)_3COCl$, NEt_3, CH_2Cl_2, 25 °C; and (iv) dealkylation, $(CH_3)_3SiBr$ (5 equiv), CH_3CN, 25 °C. A conjugate, prepared by reacting **1** with keyhole limpet hemocyanin as carrier, was used to immunize mice ($129G1X^+$ strain). Hybridoma cells were then produced by standard procedure, and mAb's of the IgG class with high titer were obtained as described.[2b]

(6) These rates were estimated at 10^{-4} to 10^{-3} s^{-1} (pH 8.0, 25 °C), an acceleration of 70- to 600-fold over background. Antibody 17G8 catalyzed the hydrolysis of **3** with $k_{cat} = 1.2 \times 10^{-4}$ s^{-1} and $K_M = 170$ μM.

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(8) Analysis performed on a RP-C18 column eluting with water:acetonitrile (82:18) at a flow of 1 mL/min with UV detector set to 254 nm. The product **4** (retention time 14 min) was collected and found to be identical by RP-HPLC coinjection and mass spectral analysis with an authentic sample that had been prepared by an alternate method and also characterized by ^{1}H NMR.

(9) Concentrations of **4** were determined by HPLC measurements of its peak height relative to that of an internal standard over 3–4 h (3 or more determinations). A standard curve showed linearity with concentrations of **4** up to 0.5 mM.

(10) Due to the high concentration of antibody used, the inhibitor and/or antibody may be depleted. The value of K_i may, therefore, be much lower than is estimated by the IC50.

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been analyzed kinetically. The hapten **1** may be considered a multi-substrate analogue of the reaction, while its phosphonyl group also simulates the characteristics of a transition state. Antibodies may be useful in distinguishing these possible attributes of enzymatic inhibitors. In the first case, the antibody would be acting as an "entropy trap",[15] while the weak binding of substrates and substrate analogues suggests it can selectively stabilize transition states. The poor binding of amides to mAb 17G8, as determined by inhibition experiments, may indicate that substrates bind in a destabilized conformation, requiring torsion about the scissile bond. The amide "resonance" makes this distortion energetically more costly for amides than for esters.[16] While **1** is not ideal as an analogue for the reaction investigated, the data show that antibody combining sites can accommodate two molecules in a chemically reactive complex at concentrations typical of enzymatic catalysis.[17] The precise mechanism of this reaction and the exploration of antibody catalysis for other bimolecular processes continue to be of interest in our investigations.[18]

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(18) This work was supported in part by grants from the NIH (GM-35318) and from the National Science Foundation (DCB-8607352). One of us (K.D.J.) thanks Igen, Inc. for financial support. Mass spectral analysis was obtained through the NSF Midwest Regional Instrumentation Facility at Lincoln, NE. We thank Diane Schloeder, Teresa Jones, and Don McLeod for expert technical assistance.

Ovothiols as Biological Antioxidants. The Thiol Groups of Ovothiol and Glutathione Are Chemically Distinct†

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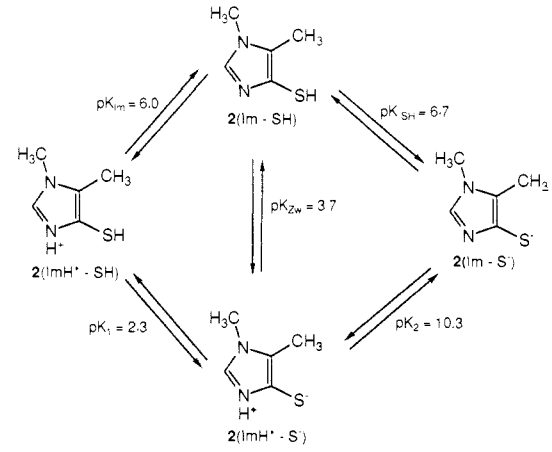
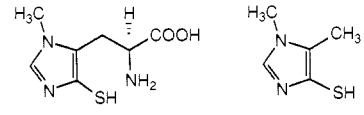
Mechanisms of action of biological antioxidants are of widespread interest because oxidative damage has been implicated in many disease states.[1] Shapiro et al. have suggested[2] that the ovothiols (e.g., **1**), a family of mercaptohistidines remarkably abundant (ca. 5 mM) in the eggs of marine invertebrates,[2,3] function as antioxidants. The presence in these eggs of both glutathione (ca. 2 mM)[4] and ovothiols suggests the possibility that these thiols may possess distinct antioxidant activities. Though the antioxidant activity of aliphatic thiols such as glutathione has been widely discussed,[5] no comparison with aromatic thiols has been made.[6] The observation[7] that the 4-mercaptoimidazole **2**[3d] is at least 50-fold superior to glutathione in inhibiting the air oxidation of pyrogallol[8] led us to investigate differences between the thiol functions of glutathione and **2**; described herein are differences in protonation state, nucleophilicity, and one-electron donating ability that are relevant to the putative role of ovothiols as antioxidants.

Scheme I





1 2

Relevant pK_a data were measured for **2**, since thiol and thiolate functions differ in reactivity. Potentiometric titration of **2** in water afforded two macroscopic pK_a's of 2.3 and 10.3; the *S*-methyl derivative of **2** yielded a single pK_a of 6.0.[9] Assuming that the latter is identical with pK_{Im} (Scheme I), the pK_a's shown in Scheme I can be calculated.[10] In sharp contrast to the thiol group of glutathione, pK_a 8.65,[11] the pK_a of the thiol of **2** is 2.3, implying that marine invertebrates are in fact 5 mM in a thiolate anion! These data indicate that **2** exists predominantly (~99.9%) as the zwitterion **2** (ImH^+-S^-) at pH 7.[12]

The predominance of an aromatic thiolate function in **2** at pH 7 suggested that the 4-mercaptoimidazoles may be more nucleophilic than glutathione. The relative nucleophilicity of **2** and glutathione in phosphate buffered water (pH 7) at 23 °C was measured by competition for a deficiency of iodoacetamide. ^{1}H NMR analysis of the resulting mixture of thioethers indicated that the rate constants for thioether formation differ by a factor of 9 in favor of **2**.[13,14] These data are in qualitative agreement with the finding that at pH 7.2 ovothiol consumes hydrogen peroxide in a second-order process 5 times as quickly as does glutathione.[2]

The most striking difference between **2** and glutathione is in the kinetics of its reactions as a one-electron donor, as might be

† This paper is dedicated to Professor E. J. Corey with best wishes on the occasion of his 60th birthday.
‡ Chevron Fellow, 1987.
§ Searle Scholar, 1984-1987; ICI Award Recipient, 1988; Fellow of the Alfred P. Sloan Foundation, 1988-1990.
(1) (a) *Oxygen Radicals and Their Scavenger Systems*; Cohen, G., Greenwald, R. B., Eds.; Elsevier: New York, 1983; Vol. I (Molecular Aspects) and Vol. II (Cellular and Medical Aspects). (b) *Pathology of Oxygen*; Autor, A., Ed.; Academic Press: New York, 1982. (c) *Oxidative Stress*; Sies, H., Ed.; Academic Press: New York, 1985. (d) *Physiology of Oxygen Radicals*: Taylor, A. E., Matalon, D., Ward, P., Eds.; American Physiological Society: Bethesda, Maryland, 1986. (e) Sies, H. *Angew. Chem., Int. Ed. Engl.* **1986**, *25*, 1058. (f) Horton, A. A.; Fairhurst, S. *CRC Critical Rev. Toxicol.* **1987**, *18*, 27. (g) Davies, K. J. A. *J. Biol. Chem.* **1987**, *262*, 9895.
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(14) The role played by each of the ionized forms of **2** and glutathione in this overall factor of 9 is at this time unknown.

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