high-throughput *ee* assays are now available to suit the needs of almost any future project. Finally, the use of directed evolution of enantioselective hybrid catalysts bearing implanted synthetic transition metal centers may open new perspectives in catalysis.[23,73]

## Acknowledgments

[73] M. T. Reetz, M. Rentzsch, A. Pletsch, and M. Maywald, *Chimia* **56,** 721 (2002).

# [22] Control of Stereoselectivity in Phosphotriesterase

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## Introduction

Phosphotriesterase (PTE), originally isolated from the soil bacterium *Pseudomonas diminuta*, catalyzes the hydrolysis of a wide range of organophosphate compounds, including agricultural insecticides and chemical warfare agents.[1] This enzyme has therefore received considerable attention as a reagent for the detoxification and remediation of organophosphorus nerve agents. However, nearly all of the most toxic chemical warfare agents, such as sarin, soman, and VX, have a stereogenic phosphorus center, and their inherent ability to inactivate the enzyme acetyl cholinesterase (AChE) varies substantially among the individual stereoisomers of these compounds. This observation indicates that the adverse biological activities of specific nerve agents are therefore highly dependent on the absolute configuration and the size of the substituents attached to the phosphorus center.

It has been shown that PTE catalyzes the hydrolysis of the insecticide paraoxon (I) at a rate that approaches the diffusion-controlled limit (Scheme 1).[2] A naturally occurring substrate for PTE has not, as yet, been identified. However, PTE has been shown to accept a substantial number of alterations in the makeup of the substituents attached to the central phosphorus core and to cleave these compounds with large catalytic rate enhancements. A fuller understanding of the overall reaction mechanism

[1] F. M. Raushel and H. M. Holden, *Adv. Enzymol. Relat. Areas Mol. Biol.* **74,** 51 (2000).
[2] D. P. Dumas, S. R. Caldwell, J. R. Wild, and F. M. Raushel, *J. Biol. Chem.* **264,** 19659 (1989).
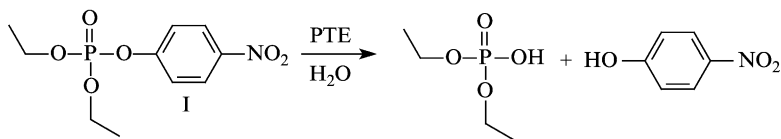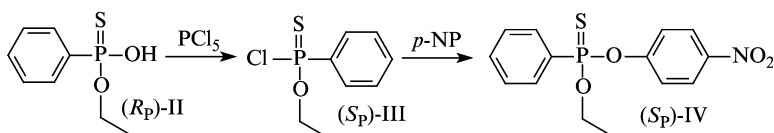
SCHEME 1.

of PTE and the elucidation of the asymmetric environment within the active site have helped to promote PTE as an effective tool for the remediation of hazardous chemical warfare agents and insecticides. Moreover, it has been demonstrated experimentally that the stereochemical constraints on substrate specificity can be manipulated systematically based on a rational reconstruction of the active site architecture.

Stereochemical Course for Substitution at Phosphorus Center

The mechanism of hydrolysis and overall stereochemistry of the reaction catalyzed by the bacterial phosphotriesterase was elucidated using $^{31}$P nuclear magnetic resonance (NMR) spectroscopy with a chiral thiophosphonate substrate.[3] It was proposed initially that the hydrolysis reaction catalyzed by PTE could proceed via one of three possible reaction mechanisms: (a) a nucleophilic aromatic substitution by water on the C-1 carbon of the leaving-group phenol that would result in the direct cleavage of the C–O bond to yield diethyl phosphate and the substituted phenol; (b) two consecutive in-line displacement reactions at the phosphorus center that would occur through the formation of a phosphorylated-enzyme intermediate and subsequent hydrolysis by an activated water molecule; and (c) a single in-line displacement reaction at the phosphorus center by a direct nucleophilic attack of an enzyme-activated water molecule. $^{31}$P NMR spectroscopy was used to distinguish mechanism a from mechanisms b and c. When paraoxon was hydrolyzed in the presence of a mixture of $^{16}$O- and $^{18}$O-labeled water, $^{18}$O was found only in the product diethyl phosphate and not in the leaving-group phenol. This experiment demonstrated unambiguously that the hydrolysis reaction occurred with P–O bond cleavage rather than an attack on the aromatic ring of the phenol leaving group.

In order to distinguish mechanism b from mechanism c, chiral versions of the insecticide O-ethyl O-p-nitrophenyl phenylphosphonothioate (EPN) were synthesized. Preliminary experiments with racemic material demonstrated that only one of the two possible enantiomers was hydrolyzed at a significant rate. The identity of the more active substrate was

[3] V. E. Lewis, W. J. Donarski, J. R. Wild, and F. M. Raushel, *Biochemistry* **27,** 1591 (1988).

Scheme 2.
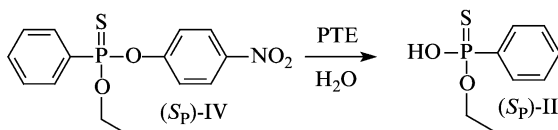
Scheme 3.

established by the separate chiral synthesis of each enantiomer. In this effort a racemic mixture of ethyl phenylphosphonothioic acid (II) was synthesized and then separated into pure enantiomers by crystallization of the 1-phenylethylamine salts. The absolute configuration of the $(R_P)$- and $(S_P)$-ethyl phenylphosphonothioic acid was determined by X-ray diffraction analysis. The $(R_P)$-O-ethyl phenylphosphonothioic acid was activated by reaction with phosphorus pentachloride to produce the corresponding chlorophosphonothioate (III), which was then reacted with p-nitrophenolate, yielding the desired product $(S_P)$-EPN (IV). The chemical transformation from $(R_P)$-O-ethyl phosphonothioic acid (II) to $(S_P)$-EPN (IV) occurs by two consecutive inversions of configuration at phosphorus, and the reactions are summarized in Scheme 2.

The overall stereochemical course of the enzymatic hydrolysis reaction catalyzed by phosphotriesterase was determined using enantiomerically pure $(S_P)$-EPN as the initial substrate. Hydrolysis of this compound by PTE provided ethyl phenylphosphonothioic acid as the sole phosphorus-containing product. The addition of $(S_C)$-1-phenylethylamine as a chiral shift reagent resulted in a ^{31}P NMR spectrum of the reaction product that was clearly superimposable upon that of an authentic sample of $(S_P)$-O-ethyl phenylphosphonothioic acid, indicating that the $S_P$ enantiomer of II was obtained by hydrolyzing $(S_P)$-EPN. These results were only consistent with a mechanism that proceeds with an overall inversion of configuration at the phosphorus center. Therefore, a phosphorylated enzyme intermediate is not formed and thus the reaction mechanism involved the direct attack of an activated water molecule on the phosphoryl center of the substrate. These reactions are summarized in Scheme 3.

Stereoselectivity of Wild-Type Phosphotriesterase

The three-dimensional X-ray structure of the Zn/Zn-substituted PTE with a bound substrate analog showed that a hydroxide from solvent and a modified lysine bridged the two metal ions within the active site. Moreover, the metal-bound hydroxide was oriented toward an in-line attack at the phosphorus center of the bound analog.[4,5] The X-ray structure of the inhibitor-bound enzyme also revealed that hydrophobic residues contributed predominantly to the formation of active site pockets. In order to delineate the structural determinants to the substrate specificity of PTE, one or both of the two ethoxy substituents of the original substrate paraoxon (I) were replaced with all possible combinations of methyl, ethyl, isopropyl, and phenyl groups (see Scheme 4).[6] The individual enantiomeric phosphotriesters were synthesized using L-proline methyl ester as a transitional chiral ligand according to published procedures.[7] Racemic mixtures and individual enantiomers were subsequently tested as substrates for the wild-type PTE by monitoring the appearance of *p*-nitrophenol at 400 nm. When racemic mixtures of phosphotriesters were hydrolyzed by PTE, an initial fast release of ∼50% of the total expected *p*-nitrophenol was produced, followed by a slower release of the remaining *p*-nitrophenol as the reaction proceeded to completion.

Although paraoxon (I) was the best substrate for the wild-type enzyme under these reaction conditions, it was found that PTE was quite tolerant of specific alterations to this basic structure. The kinetic parameters differed substantially with the size and stereochemical orientation of the substituents surrounding the phosphorus core. Moreover, the results clearly demonstrated that the regions of the active site cavity that interact with the nonleaving group substituents of the substrate played a significant role in determining the rate of substrate turnover. For all of the racemic mixtures tested within this series of compounds, the specific optical rotations for the slower substrates were always found to be for the (+)-enantiomers. The substrate reactivity of the enzyme toward the faster (−)-enantiomers of these phosphotriesters was 1- to 100-fold greater than that toward the slower (+)-enantiomers. In order to determine absolute stereochemistry for the fast and slow enantiomers, the (+)-ethyl phenyl *p*-nitrophenyl phosphate was treated with sodium methoxide using a procedure known

[4] J. L. Vanhooke, M. M. Benning, F. M. Raushel, and H. M. Holden, *Biochemistry* **35,** 6020 (1996).

[5] M. M. Benning, S.-B. Hong, F. M. Raushel, and H. M. Holden, *J. Biol. Chem.* **275,** 30556 (2000).

[6] S.-B. Hong and F. M. Raushel, *Biochemistry* **38,** 1159 (1999).

[7] T. Koizumi, Y. Kobayashi, H. Amitani, and E. Yoshii, *J. Org. Chem.* **42,** 3459 (1977).
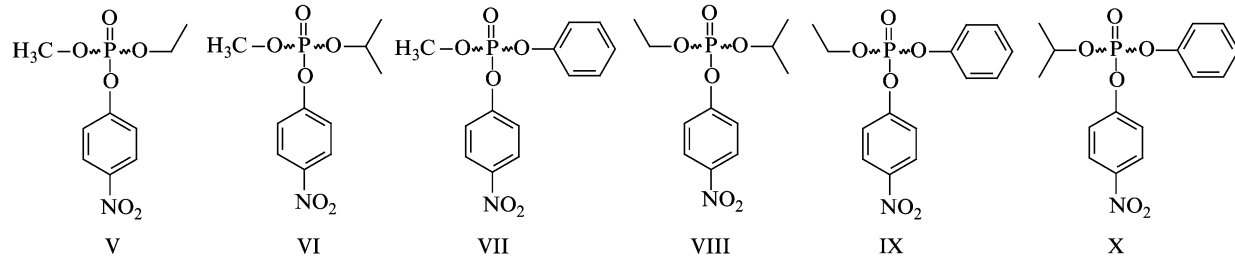
SCHEME 4.

TABLE I
RATIO OF ACTIVITIES FOR $S_P/R_P$ ENANTIOMERS OF CHIRAL
ORGANOPHOSPHATE TRIESTERS

| Compound | Enzyme | |
| :---: | :---: | ---: |
| | Wild type | G60A |
| V | 1 | 12 |
| VI | 32 | 410 |
| VII | 90 | 13,000 |
| VIII | 10 | 241 |
| IX | 21 | 11,000 |
| X | 35 | 15,000 |

to occur with an inversion of configuration at the phosphorus center. The methyl ethyl phenyl phosphate product that was formed from this transformation was determined to be of the $S_P$ configuration. This result demonstrates that the stereochemistry at the phosphorus center for the original slower organophosphate triester was of the $R_P$ configuration. Therefore, $S_P$-(−)-enantiomers are the preferred compounds for the series of substrates depicted in Scheme 4 for PTE. For the six pairs of chiral enantiomers used in this investigation, the ratios of $k_{cat}/K_m$ for the $S_P$- and $R_P$-enantiomers are presented in Table I.

Systematic Alteration of Stereoselectivity by Recombination of
Active Site Residues

A comprehensive investigation of the stereochemical constraints on the substrate specificity for PTE in conjunction with the three-dimensional structure of PTE with a bound substrate analog identified three overlapping subsites that were responsible for the binding and orientation of the substrate within the active site. These subsites for substrate binding have been designated as *small, large,* and *leaving group* pockets. These subsites (presented in Fig. 1) reflect the observed stereoselectivity for the wild-type PTE by depicting the size of the substituents as spheres of different diameters.

The amino acids that come together within the active site to form the interior walls for these three subsites were identified from the crystal structure of PTE. The *small* subsite is formed from the side chains of Gly-60, Ile-106, Leu-303, and Ser-308. The *large* subsite contains His-254, His-257, Leu-271, and Met-317, whereas the residues that form the *leaving group* subsite are Trp-131, Phe-132, Phe-306, and Tyr-309. Systematic amino acid substitutions within the small pocket, the large pocket, and
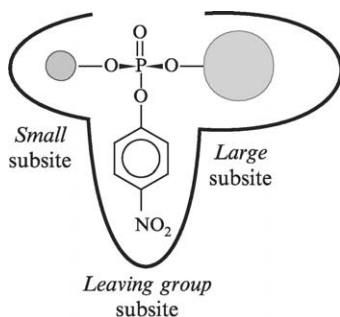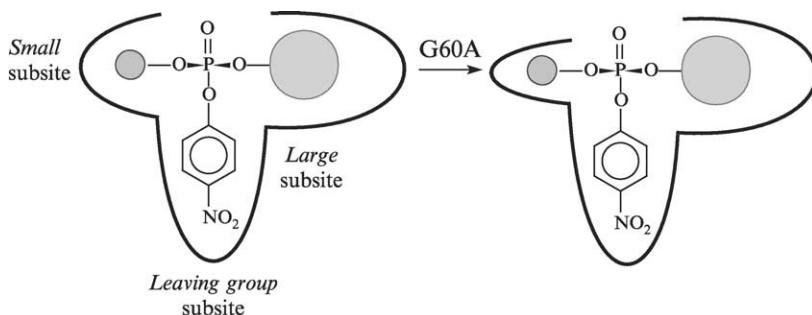
FIG. 1. The substrate-binding cavity of PTE.



FIG. 2. Constriction of the small subsite to enhance stereoselectivity.

the leaving group pocket generated a small library of enzyme mutants that were characterized for alterations in the substrate profiles using the small library of modified organophosphate triesters.[8,9] The initial objective was to determine if the observed stereoselectivity of the wild-type enzyme could be *enhanced* without having to compromise the substantial turnover rates for the initially preferred enantiomer. It was anticipated that this design feature could be realized by constricting the size of the small pocket. Substantial success was achieved with the replacement of Gly-60 with a single alanine residue as presented in Fig. 2. The relative kinetic parameters for the G60A mutant with the six pairs of racemic substrates that appear in Scheme 4 are presented in Table I. For these substrates the observed stereoselectivity has been enhanced up to 500-fold. This mutant

[8] M. Chen-Goodspeed, M. A. Sogorb, F. Wu, S.-B. Hong, and F. M. Raushel, *Biochemistry* **40,** 1325 (2001).
[9] M. Chen-Goodspeed, M. A. Sogorb, F. Wu, and F. M. Raushel, *Biochemistry* **40,** 1332 (2001).

is able to distinguish between a methyl and an ethyl substituent by about an order of magnitude. This mutant has proven to be quite effective in the kinetic resolution of racemic organophosphates on a preparative scale.

Relaxation of the stereoselectivity observed with the wild-type PTE was achieved by enlargement of the small subsite in order to accommodate bulkier substituents within the active site of the protein. Replacement of Ile-106, Phe-132, and/or Ser-308 with alanine and/or glycine residues resulted in the loss of the chiral preference for the initially preferred $S_P$-enantiomers. These substitutions appreciably increased the rate of phosphotriesterase for the initially slower $R_P$-enantiomers. For example, with the I106G mutant the $k_{cat}/K_m$ values for compounds IV and VII increased by factors of 74 and 35, respectively. For the I106G/S308G mutant, the value of $k_{cat}/K_m$ for compound IX increased from $5.2 \times 10^6$ to $2.2 \times 10^8 \ M^{-1} \ s^{-1}$.

The most difficult transformation attempted was the reversal of the stereoselectivity exhibited by the wild-type enzyme. This required enhancement in the rate of turnover for the initially slower $R_P$-enantiomers while simultaneously reducing the rate of turnover for the initially faster $S_P$-enantiomers. The engineering strategy for this objective is presented in Fig. 3 and involved the enlargement of the small subsite and reduction in the size of the large subsite. In order for this plan to be effective, specific alterations to the active site had to be additive with one another. The expansion of the small subsite had already demonstrated that the initially slower $R_P$-enantiomers could be made to turn over at a faster rate. A mutagenic survey of those residues within the large subunit identified His-254 and His-257 as the most likely candidates for contraction of the large subsite via substitution with bulkier side chains. For example, with the mutant H257Y, the value of $k_{cat}/K_m$ for $S_P$-VII decreased by a factor
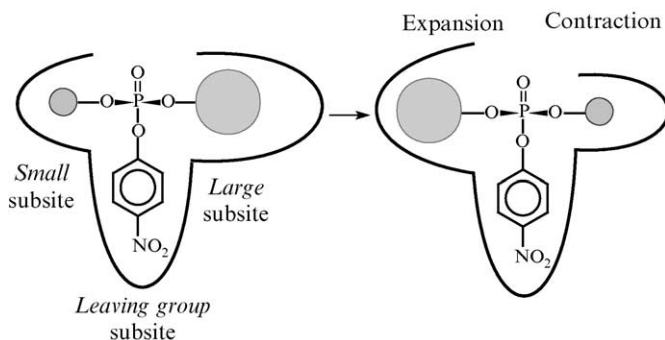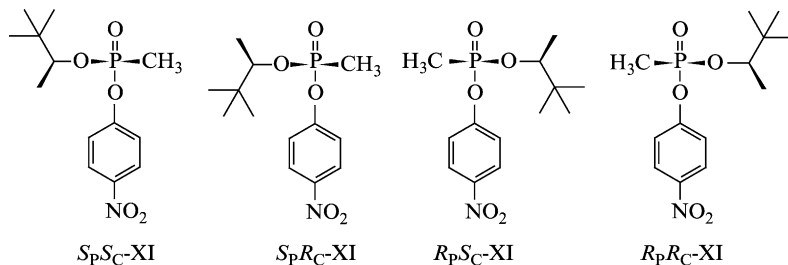


Fig. 3. Strategy for the reversal of stereoselectivity.

$S_PS_C$-XI          $S_PR_C$-XI          $R_PS_C$-XI          $R_PR_C$-XI

SCHEME 5.

of 20, relative to the wild-type enzyme, but was essentially unchanged for the corresponding $R_P$-enantiomer. When this mutation (H257Y) was combined with alterations to the small subsite, a reversal in stereoselectivity was achieved for most of the racemic mixtures presented in Scheme 4. With the mutant I106G/F132G/S308G/H257Y the $R_P$-enantiomers are preferred over the $S_P$-enantiomers by factors of 190, 80, and 460 for compounds VII, IX, and X, respectively.

These results demonstrated quite clearly that significant alterations to the relative substrate profiles could be achieved with a small number of amino acid changes that are located exclusively within the active site. The most dramatic differences in stereoselectivity were achieved with compound X. The G60A mutant exhibited a preference for the $S_P$-enantiomer over the $R_P$-enantiomer by a factor of 15,000, whereas the I106G/F132G/S308G/H257Y mutant had a preference for the $R_P$-enantiomer over the $S_P$-enantiomer by a factor of 460. Between these two extremes there is nearly a seven orders of magnitude change in the observed substrate preference for one enantiomer over the other!

Another approach has proven to be effective in the identification of mutants of PTE that are improved for the hydrolysis of specific organophosphates nerve agents.[10] Scheme 5 shows the structures of four stereochemical analogs (XI) of the nerve agent soman. The toxicity of soman and the analogs shown in Scheme 5 are very much dependent on the stereochemistry at the chiral phosphorus and carbon centers. Unfortunately, the $S_PS_C$ isomer is the most toxic but the poorest diastereomeric substrate for the wild-type PTE. In an effort to improve the turnover rate for the most toxic isomer, two amino acid positions within the wild-type sequence (His-254 and His-257) were randomized to all possible combinations of the 20 amino acids (for a total of 400 possible mutants). This library

[10] C. R. Hill, W.-S. Li, J. B. Thoden, H. M. Holden, and F. M. Raushel, *J. Am. Chem. Soc.* **125,** 8990 (2003).

of mutants was screened in a whole cell format and a single mutant stood out from the rest, H254G/H257W. This mutant was two orders of magnitude better than the wild-type enzyme for the hydrolysis of $S_P S_C$-XI. This mutant was subsequently used as the parent enzyme, and L303 was replaced with all 20 natural amino acids. Of these mutants the substitution with threonine produced another order of magnitude increase in substrate turnover for the target substrate. The H254G/H257W/L303T mutant was improved in the value of $k_{cat}/K_m$ by nearly three orders of magnitude. Again these studies demonstrated that a relative small number of mutations that were focused exclusively within the active site could elicit significant improvements in enzymatic turnover for specific target substrates.

Enzymatic Syntheses of Chiral Organophosphate
   and Organophosphothioates

Most of the published work on the chemical synthesis of chiral phosphotriesters has involved the use of stereospecific alcoholysis of an optically active intermediate or treatment with a chiral-resolving ligand, resulting in lengthy chemical procedures and the very time-consuming chiral separation of diastereomers.[7,11] The toxicity of many organophosphates depends on the chirality at the phosphorus center. Therefore, it is not surprising that much attention has been paid to the development of more efficient preparations of pure enantiomeric organophosphorus compounds for use as enzyme substrates and inhibitors. New approaches to the synthesis of chiral organophosphorus compounds have been developed using the engineered mutants of phosphotriesterase that facilitate the kinetic resolution of racemic mixtures of organophosphate triesters.[12] This approach is a direct consequence of the ability of the mutant G60A to leave the slow $R_P$-enantiomers intact when presented with a racemic mixture of phosphotriesters.[8] Likewise, hydrolysis of racemic mixtures of phosphotriesters catalyzed by the mutant I106G/F132G/H257Y enable the isolation of the corresponding $S_P$-enantiomers.[9] Treatment of racemic organophosphate triesters in 100 m$M$ CHES buffer (pH 9.0) containing 5–20% acetonitrile with the G60A mutant enzyme yielded $R_P$ triesters with >95% enantiomeric excess and isolate yields of >70%. When racemic mixtures of phosphotriesters were hydrolyzed by the I106A/F132A/H257W mutant, the $S_P$-enantiomers were obtained with >96% enantiomeric excess with >60% yield.

[11] C. R. Hall, T. D. Inch, G. J. Lewis, and R. A. Chittenden, *J. Chem. Soc. Chem. Commun.* 720 (1975).
[12] F. Wu, W.-S. Li, M. Chen-Goodspeed, M. A. Sogorb, and F. M. Raushel, *J. Am. Chem. Soc.* **122,** 10206 (2000).
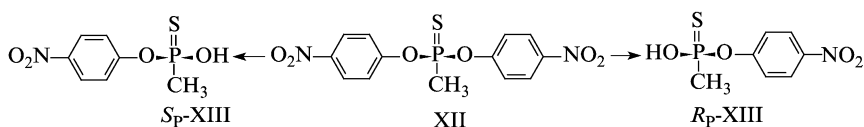
SCHEME 6.

The library of PTE mutants has also been exploited for the preparation of chiral organothiophosphates from prochiral starting materials.[13] For example, enzymatic hydrolysis of bis-*p*-nitrophenyl methyl phosphothioate (XII) would form the $R_P$ isomer of XIII via cleavage of the *pro*R substituent and the $S_P$ isomer of XIII via cleavage of the *pro*S substituent, as the stereochemical course of the reaction is known to proceed with a net inversion of configuration. These reaction products are illustrated in Scheme 6. Product stereochemistry was confirmed by $^{31}$P NMR spectroscopy with a chiral shift reagent and chemical transformation to products of known stereochemistry. The wild-type PTE was found to cleave the *pro*R substituent as predicted by analogy with prior stereochemical investigations.[6] The prochiral stereoselectivity catalyzed by the selected engineered mutants of PTE was reversed due to the alterations in the size of the substrate-binding cavity. The mutant I106A/F132A/H257W was found to cleave the *pro*S substituent and thus reversed the stereoselectivity of the wild-type enzyme for the substrate bis-*p*-nitrophenyl methylphosphonate compared with the wild-type PTE.

## Concluding Remarks

The active site of the bacterial phosphotriesterase has served as a predictable template for the modulation of substrate and stereoselectivity via site-directed mutagenesis. In this regard the stereoselectivity of PTE has been *enhanced, relaxed,* and *reversed* with remarkably few changes in the overall amino acid sequence. These modified proteins have proven to be of practical utility in the preparative isolation of chiral phosphorus products via the kinetic resolution of racemic mixtures.

## Acknowledgments

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[13] W.-S. Li, Y. Li, C. M. Hill, K. T. Lum, and F. M. Raushel, *J. Am. Chem. Soc.* **124,** 3498 (2000).