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Inhibition of *Helicobacter pylori* Urease by Phenyl Phosphorodiamidates: Mechanism of Action

W. Stephen Faraci, Bingwei V. Yang, Dawn O'Rourke and Robin W. Spencer
Medicinal Chemistry Department, Pfizer Central Research, Groton, CT 06340, U.S.A.

Abstract—*Helicobacter pylori* urease is a nickel-containing enzyme that hydrolyzes urea to bicarbonate and ammonia. Andrews *et al.* (*J. Am. Chem. Soc.* **1986**, *108*, 7124)[1] have shown that amides and esters of phosphoric acid are slow, tight-binding inhibitors of urease isolated from jack bean. We show that 4-substituted phenyl phosphorodiamidates (4-R-PhOP(=O)$(NH_2)_2$) are slow-binding inhibitors of *H. pylori* urease with no evidence of kinetic saturation. Their second-order rates of inhibition k_1 are strongly correlated with phenol pK_a (e.g. R = NO_2, $k_1 = 2.5 \times 10^5$ $M^{-1}s^{-1}$; R = OMe, $k_1 = 1.2 \times 10^4$ $M^{-1}s^{-1}$). The Bronsted β for inhibition is 0.4, similar to that of model system $S_N2(P)$ reactions. Based on these observations, we suggest that urease inhibition is covalent but reversible, involving a common phosphoacyl enzyme intermediate.

Introduction

Helicobacter pylori, a Gram negative, microaerophilic spiral bacterium, is strongly associated with peptic ulcers and gastric cancer.[2–6] *Helicobacter pylori* is acid sensitive and replicates in a pH range of 7–8, but is adapted for survival in the highly acidic environment of the human stomach. It produces a highly active urease which hydrolyzes urea (from the host bloodstream) to produce bicarbonate and ammonia. Brady *et al.*[7] suggest that urease activity is essential for *H. pylori* survival by generating a neutralizing base (ammonia) to counteract stomach acid. It is also postulated that urease activity may act as virulence factor for *H. pylori* and contribute to gastric mucosal damage.[8,9]

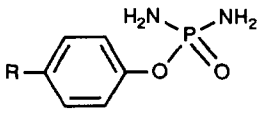
Urease has been isolated and purified to homogeneity from *H. pylori*,[4] and has two subunits of molecular weight 66 and 29.5 kDa. The enzyme is an $\alpha_6\beta_6$ complex with a native molecular weight of 550 kDa. N-Terminal analysis of *H. pylori* urease shows that it is very similar to urease isolated from uropathogenic bacterial species and the jack bean.

Phenyl phosphorodiamidates are potent inhibitors of urease isolated from soil bacteria.[10–12] Andrews *et al.*[1] have shown that amides and esters of phosphoric acid inhibit urease isolated from jack bean. They show that phenyl phosphorodiamidate **3** is a slow, tight-binding inhibitor of jack bean urease and suggest that inactivation proceeds by initial hydrolysis of phenyl phosphorodiamidate to give tightly bound phosphorodiamidic acid **6**.

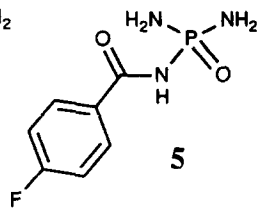
We show here that phosphorodiamidates **1–6** are potent, slow-binding inhibitors of *H. pylori* urease. For the phenol esters **1–4**, the strong correlation of inhibition rate with phenol leaving group potential suggests that P–O–(aryl) bond scission is rate determining in inhibition. This could be due either to enzyme-catalyzed phosphorodiamidic acid production as Andrews *et al.* suggest, or to inhibition by a phosphoacyl intermediate. Since both phosphorodiamidic acid **6** itself and flurofamide **5** lie on the correlation with the phenol esters, we suggest a common mechanism for all these inhibitors which includes a phosphoacyl enzyme intermediate.

Results

Previous work has suggested that phenyl phosphorodiamidate **3** is a slow, tight-binding inhibitor of bacterial[11] and jack bean urease.[1] However, the



1 R = NO_2
2 R = Cl
3 R = H
4 R = OCH_3



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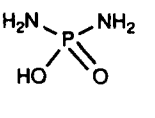

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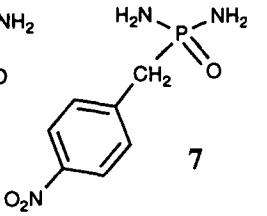

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kinetics of inhibition have not been fully elucidated. As shown in Figure 1a, addition of phosphorodiamidic acid **6** to an enzyme–substrate assay mixture leads to a first order decrease in the rate of urea hydrolysis. Similar slow-binding inhibition was observed for compounds **1–5**. Additional information on the mechanism of inhibition can be obtained by plotting the rate of approach to the steady state of urease inhibition (k_{obs}) as a function of inhibitor concentration.

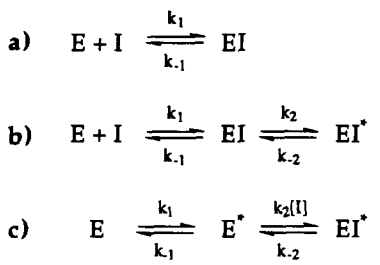
As shown in Figure 1b, a plot of k_{obs} versus [I] for **6** is linear to [I] ≈ 12 μM; there is no evidence of saturation. This linearity is also seen with **1–4**. As described in Bakker *et al.*,[18] there are three possible kinetic mechanisms which describe slow-binding inhibitors (Scheme 1). The rates of approach to the new steady state (k_{obs}) predicted by mechanisms A, B and C in Scheme 1 are given by equations 1, 2 and 3 respectively,[21] in the limit [S] << K_m under which these experiments were performed ([S] = 200 μM, K_m = 1 mM):

$$k_{obs} = k_{-1} + k_1[I] \quad (1)$$

$$k_{obs} = k_{-2} + k_2[I]/([I] + K_i) \quad (2)$$

$$k_{obs} = k_1 + k_{-1}K_i/([I] + K_i) \quad (3)$$

Two-state inhibition (Scheme 1a) is the simplest kinetic mechanism consistent with our data in which $k_{obs} = k_{-1} + k_1[I]$.



Scheme 1.

The slopes of k_{obs} versus [I] provide k_1 values (Table 1) for each phosphorodiamidate. These k_1 values show that inhibitors containing a good leaving group (4-nitrophenol, 4-chlorophenol) have fast inhibition rate constants while those compounds with poorer leaving groups (phenol, 4-methoxyphenol, *p*-fluorobenzamide) have slower rates. A plot of leaving group ability (as judged by the pK_a of the leaving group) versus inactivation rate (k_1) is shown in Figure 2; the slope indicates a Bronsted β of 0.37 ± 0.06. Incubation of **7** (the carbon analog of **1**) with enzyme did not display any measurable inhibition up to 32 μM, consistent with elimination of the leaving group as an essential step in enzyme inhibition.

To obtain a value for K_i, the dissociation rate of the enzyme–inhibitor complex (k_{-1}) must be determined. Unfortunately, return of activity experiments were not successful with phosphorodiamidates. Aliquots of the urease–phosphorodiamidate complex led to a small (*ca* 10%) increase in activity after 24 h as compared to control enzyme. Since virtually all enzyme activity in the control sample was gone by 48 h, a value for k_{-1} could not be obtained.

Discussion

Andrews *et al.*[1] reported that esters and amides of phosphoric acid inhibit jack bean urease. These authors propose that the inhibitory species is phosphorodiamidic acid **6**, which inhibits by forming a stoichiometric complex with the active site nickel ion (path a in Scheme 2). Hydrolysis of the phenyl ester is assisted enzymically, producing the phosphorodiamidic acid–nickel inhibitor complex (path b in Scheme 2). Thus, addition of phosphorodiamidic acid **6** alone should lead to rapid inhibition of urease. Alternatively, an enzyme nucleophile may attack phenyl phosphorodiamidate to form a phosphoacyl enzyme intermediate (Scheme 3).

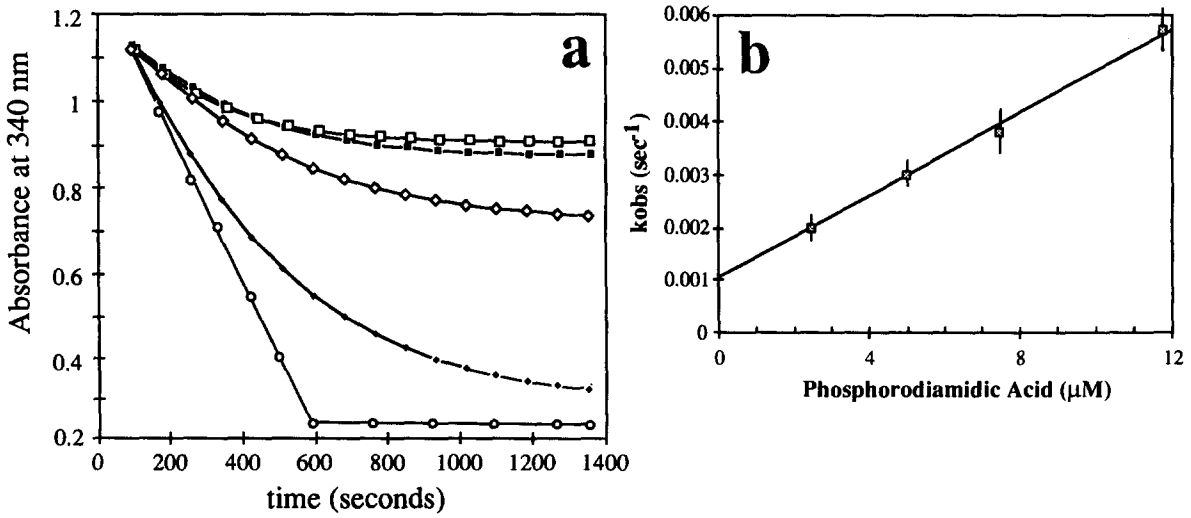


Figure 1. (a) Progress curves of *H. pylori* urease inhibition by (○) 0 μM, (◆) 2.5 μM, (◊) 5 μM, (■) 7.5 μM, and (□) 12 μM phosphorodiamidic acid **6** in assays containing 100 μM substrate. (b) Rate of approach to the steady state of *H. pylori* urease inhibition (k_{obs}) as a function of phosphorodiamidic acid concentration.

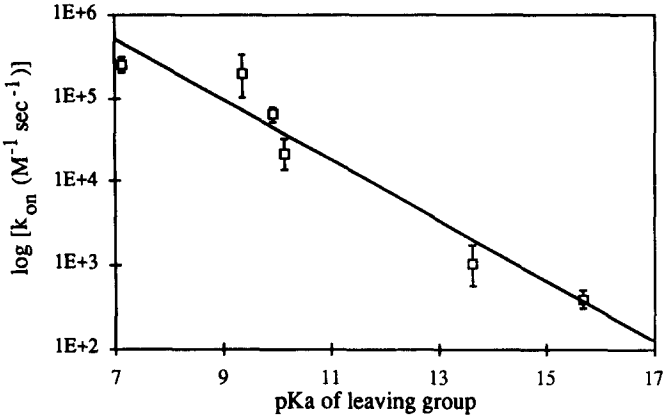
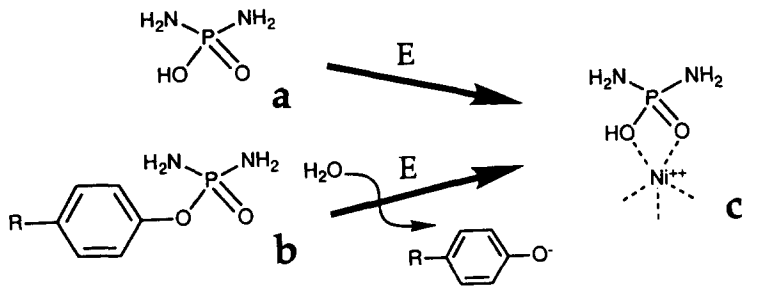


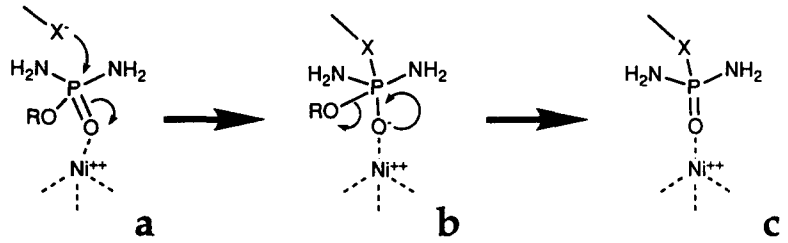
Figure 2. Linear free-energy relationship between the rate constant for inhibition (k_1) of *H. pylori* urease by compounds **1–6** and the pK_a of the conjugate acids of the leaving groups (Table 1). The slope of the best fit line (= $-\beta$) is 0.37 ± 0.06.

Table 1.

Compound	Leaving group	pK_a	k_1 ($M^{-1}s^{-1}$)
1	4-NO_2-phenol	7.14	$2.5 \pm 0.2 \times 10^5$
2	4-Cl-phenol	9.38	$2.0 \pm 1 \times 10^5$
3	phenol	9.95	$5.5 \pm 1 \times 10^4$
4	4-OMe-phenol	10.20	$2.5 \pm 1 \times 10^4$
5	4-F-benzamide	13.7	$1.0 \pm 0.5 \times 10^3$
6	hydroxide	15.7	$3.8 \pm 0.5 \times 10^2$
7	4-NO_2-benzyl	>20	$< 1 \times 10^2$



Scheme 2.



Scheme 3.

In this case the rate of inhibition should depend on the rate of elimination of the leaving group.

From the data presented in Table 1 and Figure 2, we propose that the probable inhibited species is such a covalent phosphoryl enzyme. If phosphorodiamidic acid **6** was the direct inhibitory species, the inactivation rate should be *greater* for **6** than any phenyl ester (**1–5**) since **6** does not have to undergo hydrolysis. However, **6** inhibits urease much more slowly than compounds **1–5** and in fact correlates well with the prediction that elimination of the leaving group (= OH^- for **6**) determines inhibition rate. The phosphoenzyme hypothesis is further supported by inhibition rates of flurofamide (**5**) and *p*-nitrobenzylphosphonic diamide **7**. Flurofamide (**5**), which contains a fluorobenzamide leaving group (p$K_a \approx$ 13.7), inactivates urease with $k_1 = 1 \times 10^3$ $M^{-1}s^{-1}$, again correlating with the phenyl phosphorodiamidate data. No inhibition of *H. pylori* urease activity was observed upon incubation of *p*-nitrobenzylphosphonic diamide (**7**) at concentrations up to 30 μM. Since 4-nitrobenzyl

carbanion ($pK_a >> 20$) or the NH_2- anion ($pK_a = 33$) are much poorer leaving groups than phenols or benzamides, one would not expect to see enzyme inactivation if elimination were necessary for inhibition. Because we cannot measure inhibition rates less than 100 $M^{-1}s^{-1}$, we would not expect to observe inhibition by compounds with leaving group pK_a greater than 18.

Given the correlation of Figure 2, what does the *value* of the slope suggest by comparison to model system chemistry? Williams and Douglas [20] measured the rate constants for alkaline hydrolysis (k_{OH^-}) of phenyl phosphorodimorpholidate and *N,N'*-diphenyl phosphorodiamidate and plotted this against pK_a of the leaving group, obtaining $\beta = 0.85$. Based on the Hammett relationship, linear free-energy relationship and the rate constant for base hydrolysis between the two, they concluded that *N,N'*-diphenyl phosphorodiamidate hydrolysis proceeds via an E1cB mechanism while phenyl phosphorodimorpholidate hydrolysis (with $\beta = 0.42$) proceeds via $S_N2(P)$-like mechanism.[23] A Bronsted β for urease inactivation by phosphorodiamidates was calculated from Figure 2 and shown to be 0.4, very similar to the Bronsted β for phenyl phosphorodimorpholidate hydrolysis, providing further evidence for an $S_N2(P)$-like inactivation mechanism.

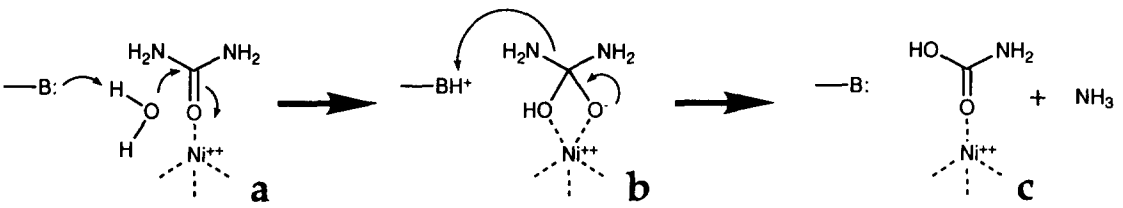
Thus, the simplest mechanism that best represents the data is attack of an enzyme nucleophile to yield an enzyme–phosphate complex concomitant with elimination of the leaving group (Scheme 3). Since all enzyme–inhibitor complexes (intermediate **c** in Scheme 3) are the same, k_2 should be similar. Unfortunately, we were unable to obtain k_2 values for compounds **1–6** due to the extremely long reactivation rates of the enzyme–inhibitor complex and the inherent enzyme instability at 37 °C. Based on these data, it is also possible to speculate on the mechanism of enzymatic urea hydrolysis. Two plausible mechanisms are (1) direct hydrolysis of the urea carbonyl group, yielding a hemiketal that breaks down to ammonia and carbamate (Scheme 4) or (2) attack of an enzyme nucleophile on the urea carbonyl group to give an acyl enzyme intermediate which hydrolyzes to give carbamic acid (Scheme 5). Unlike the zinc metalloprotease hydrolysis which involves direct attack of water on the peptide bond, we propose that urea catalysis occurs through formation of an acyl–enzyme intermediate, similar to the mechanism of inactivation by phosphorodiamidates.

In summary, we have shown that phenyl phosphorodiamidates are time-dependent, slow-binding inhibitors of *H. pylori* urease. A Bronsted plot of the data from Table 1 (Figure 2) suggests that inhibition is not simply tight binding of phosphorodiamidate to the enzyme (Scheme 2) but rather nucleophilic attack of the enzyme to yield a covalent phosphoryl intermediate (Scheme 3) which slowly dissociates. We are presently investigating this mechanism in more detail.

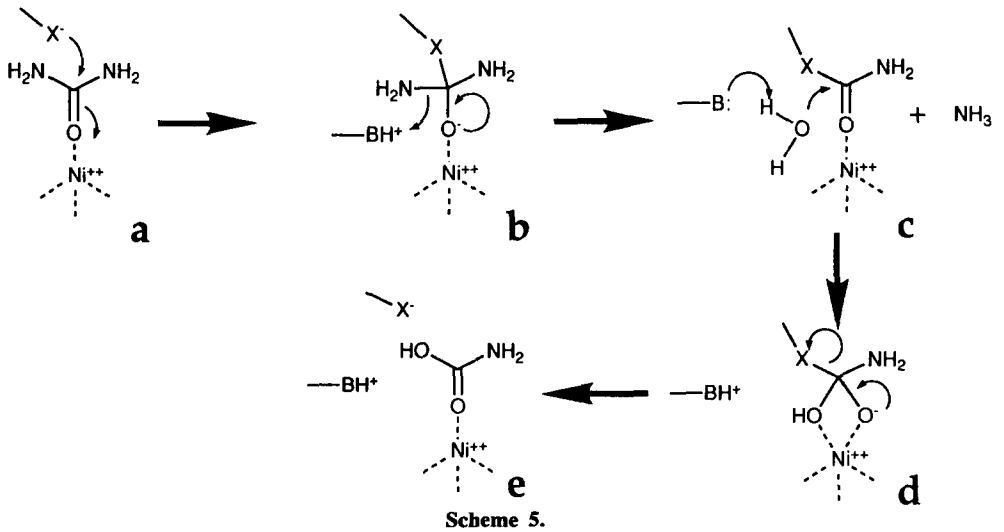
Experimental

Materials

HEPES, urea and 2-ketoglutaric acid were purchased from Sigma Chemical Co., St. Louis, MO; glutamate



Scheme 4.



Scheme 5.

dehydrogenase and NADH were purchased from Boehringer–Mannheim. *Helicobacter pylori* urease was purchased from Dr H. Mobley of the University of Maryland School of Medicine, Baltimore, MD. The enzyme was partially purified and had a specific activity of 145 units mL^{-1}.

Synthesis of phosphorodiamidates

Phenyl phosphorodiamidate **3** was purchased from Fairfield Chemical Co. Compounds **1**, **2** and **4** were prepared by reaction of the corresponding phenyl phosphorodichloridate with either ammonia or ammonium hydroxide.[13] A typical procedure for the preparation of compound methoxyphenyl phosphorodiamidate **4** is as follows: 4-methoxyphenol (10.5 g, 81 mmol) was dissolved in $POCl_3$ (250 mL) at 0 °C. To this solution was added aluminum chloride (1.17 g, 8.76 mmol) at 0 °C. The reaction mixture was heated at reflux overnight. After removal of $POCl_3$, the obtained 4-methoxyphenyl phosphorodichloridate was dissolved in $CHCl_3$ (300 mL). The solution was cooled to −10 °C and saturated with ammonia gas for 35 min. The mixture, after standing at room temperature for 1 h, was filtered and the solid on the funnel was thoroughly washed with ice water to remove the salt. The residue was crystallized from hot water and methanol to give 6.9 g of 4-methoxyphenyl phosphorodiamidate **4** as a white solid, mp 193–195 °C. ^{1}H NMR (DMSO-d_6) 7.09 (*d*, *J* = 9.0 Hz, 2H), 6.86 (*d*, *J* = 9.0 Hz, 2H), 4.27 (*d*, *J* = 4.0 Hz, 4H), 3.72 (*s*, 3H). Anal. calcd for $C_7H_{11}N_2O_3P$: C, 41.59; H, 5.48; N, 13.86. Found: C, 41.36; H, 5.24; N, 13.80.

4-Nitrophenyl phosphorodiamidate **1** was synthesized as above using 4-nitrophenol to give a 19% yield, mp 182–184 °C. ^{1}H NMR (DMSO-d_6) 7.37 (*d*, *J* = 8.6 Hz, 2H), 7.20 (*d*, *J* = 8.6 Hz, 2H), 4.41 (*d*, *J* = 3.3 Hz, 4H). FAB-MS: m/z 218 (MH^+). Anal. calcd for $C_6H_8N_3O_4P$: C, 33.19; H, 3.71; N, 19.35. Found: C, 33.22; H, 3.75; N, 19.14.

4-Chlorophenyl phosphorodiamidate **2** was synthesized as above using 4-chlorophenol to give a 2.4% yield, mp 171–173 °C. ^{1}H NMR (DMSO-d_6) 7.37 (*d*, *J* = 8.6 Hz, 2H), 7.20 (*d*, *J* = 8.6 Hz, 2H), 4.41 (*d*, *J* = 3.3 Hz, 4H). FAB-MS: m/z 207 (MH^+).

4-Nitrobenzylphosphonic diamide **7** was prepared by reaction of 4-nitrobenzylphosphonic dichloride with ammonia in a similar manner as above.[14] A sample of 1.3 g of 4-nitrobenzylphosphonic dichloride afforded 0.678 g (79% yield) of 4-nitrobenzylphosphonic diamide **7** as a white solid, mp 173–175 °C. ^{1}H NMR (DMSO-d_6) 8.13 (*d*, *J* = 8.5 Hz, 2H), 7.51 (*d*, *J* = 8.5 Hz, 2H), 3.78 (*br s*, 4H), 3.1 (*d*, *J* = 19.9 Hz, 2H). FAB-MS: m/z 216 (MH^+).

Phosphorodiamidic acid **6** was prepared according to the method of Klement *et al.*,[15] and flurofamide **5** was prepared according to the literature procedure.[16]

Analytical methods

Helicobacter pylori urease activity was measured in 0.8 mL of 0.02 M HEPES buffer (pH 7.2, 37 °C) containing 10 mM 2-ketoglutarate, 40 μL of 25 mg mL^{-1} glutamate dehydrogenase, 250 μM NADH and 1 mM urea. Activity was measured by following the resulting decrease in absorbance at 340 nm ($\Delta\varepsilon$ 6220 $M^{-1}cm^{-1}$). Slow-binding inhibition[17] was apparent in the progress curves of inhibition by each compound under assay conditions in which urea concentration (200 μM) is less than its K_m. Concentrations of 5–20 nM inhibitor were used with inhibitors **1** and **2**, 10–75 nM for **3**, 50–500 nM for **4**, 100–500 nM for **5**, 2.5–12.5 μM for **6** and 3–32 μM for **7**. Each progress curve was fitted to the single exponential equation $y = Ae^{-kt} + B$ by non-linear least squares regression.[18,19] In all cases the steady state (final) rate was indistinguishable from zero when the data were fitted to $y = Ae^{-kt} + B + Ct$.

Return of activity

To determine if the inhibition of urease by phosphorodiamidates **1–6** is reversible, activity was assayed upon dilution of the enzyme–inhibitor complex such that the excess inhibitor level was well below the enzyme concentration. Urease solution was inhibited with 5 μM **1–6**, excess inhibitor diluted by repeated passing through a Centricon 30 filtration apparatus, and the solution placed in a 37 °C water bath. At various time intervals, a 10 μL aliquot was diluted into 0.8 mL assay buffer containing substrate (2 mM) and coupling enzymes, and activity measured as a function of time. A control rate was also measured in which enzyme was incubated in the absence of inhibitor and an aliquot diluted into an assay cuvette.

Acknowledgements

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