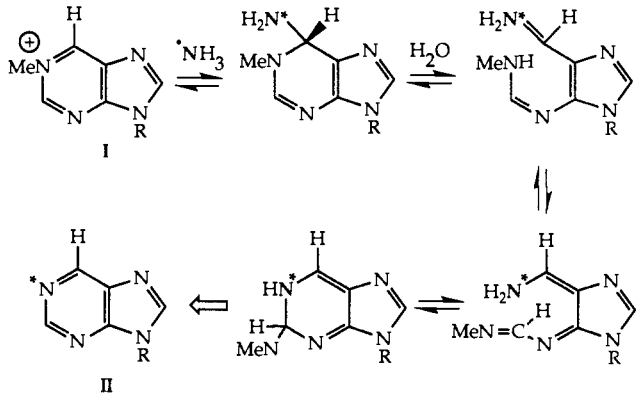
Scheme I



deaminase binds this covalent hydrate, believed to resemble an unstable intermediate in substrate hydrolysis, with an extremely high affinity approaching that expected of an ideal transition state analogue inhibitor,[1] and to show remarkable powers of discrimination between this and closely related compounds.[5] ^{15}N-labeled purine ribonucleoside may prove useful in investigating the structural details of this and other enzyme–ligand interactions.

Acknowledgment. We are grateful to the North Carolina Biotechnology Center for an undergraduate research fellowship (to J.A.) supported by a grant to the Program in Molecular Biology and Biotechnology and to the National Institutes of Health (Grant No. GM-18325) for support of this work.

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Phosphoenolpyruvate as a Natural Bisubstrate Analogue Inhibitor of Pig Kidney Prolidase

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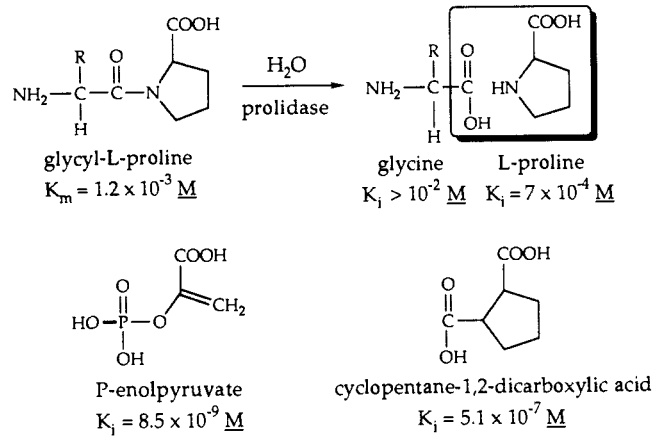
Among the few enzymes that are known to catalyze hydrolysis of peptide bonds involving the nitrogen atom of proline are prolidase (EC 3.4.13.9), a manganese-dependent dipeptidase that cleaves substrates of the type X-Pro,[1] and several virally encoded endoproteases that are responsible for processing the gene products *gag* and *pol* in HIV-1 and other retroviruses.[2] Prolidase is present in microorganisms and most mammalian tissues, where it permits terminal degradation of exogenous or endogenous polypeptides, and humans deficient in this enzyme suffer from skin lesions and limb abnormalities.[3] It has been suggested that prolidase may be involved in the intracellular metabolism of procollagen[4] and that, since the enzyme is cytosolic, a potent in vivo inhibitor should be capable of penetrating cell membranes.[5]

With a view to developing strong competitive inhibitors of pig kidney prolidase, we sought to combine structural features of the

Scheme I



two substrates for reversal of dipeptide hydrolysis within a single molecule, expecting that the resulting multisubstrate analogue inhibitor might exhibit a substantially higher binding affinity than the combined affinities of the two substrates.[6] At their moment of inception, the combined products of hydrolysis of a typical dipeptide substrate, Gly-Pro, seem likely to be arranged as shown in Scheme I. A similar arrangement would then be present at the active site when these compounds acted as substrates for the reverse, peptide bond forming, reaction. Compounds such as 1,2-cyclopentanedicarboxylic acid, bearing some structural resemblance to this arrangement (Scheme I), might then serve as effective inhibitors of prolidase in vitro.

For convenience in examining potential inhibitors,[7] we developed a continuous spectrophotometric assay, following the disappearance of the substrate Gly-Pro at 222 nm ($\Delta\epsilon_m = -904$) in K^+-MES buffer (0.01 M pH 6.0) at 20 °C. Double reciprocal plots of initial reaction velocities against substrate concentration, determined in the presence and absence of inhibitors, were linear, intersecting at the ordinate as expected for competitive inhibition. *trans*-1,2-Cyclopentanedicarboxylic acid exhibited a K_i value (5.1×10^{-7} M) much lower than the K_m value observed for the substrate Gly-Pro (1.7×10^{-3} M) and considerably lower than K_i values reported for the strongest inhibitors described previously, Cbz-Pro (9×10^{-5} M) and pyrrolidine-2-phosphonic acid (9.5×10^{-4} M).[5,8,9]

When other potential inhibitors were examined, we were surprised to find that the activity of 1,2-cyclopentanedicarboxylic acid was surpassed by that of the well-known glycolytic intermediate P-enolpyruvate, a competitive inhibitor with a K_i value of 8.5×10^{-9} M. Scheme I shows that P-enolpyruvate exhibits several structural features expected of a multisubstrate analogue, if its phosphoryl group is considered to replace one of the carboxyl groups of the two substrates for peptide-bond formation,[10] and its vinyl group projects into a hydrophobic cavity that might normally be occupied by the pyrrolidine ring of proline.

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(7) Earlier assays of prolidase were based on analysis of reaction mixtures at timed intervals, for example, by colorimetric methods (Chinard, F. P. *J. Biol. Chem.* **1952**, *199*, 265. Mayer, H.; Nordwig, A. *Hoppe-Seyler's Z. Physiol. Chem.* **1973**, *354*, 371) or by proton magnetic resonance spectroscopy (King, G. F.; Middlehurst, C. R.; Kuchel, P. W. *Biochemistry* **1986**, *25*, 1054).
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(9) Captopril has been reported to give slightly stronger inhibition ($K_i = 3 \times 10^{-5}$ M) of rat kidney and liver prolidases (Ganapathy, V.; Pashley, S. J.; Roesel, R. A.; Pashley, D. H.; Leibach, F. H. *Biochem. Pharmacol.* **1985**, *34*, 1287), but this was not observed for the pig kidney enzyme by King et al.[5] We have confirmed the findings of the latter investigators.
(10) The phosphoryl group of P-enolpyruvate could be superimposed on either of the original carboxyl groups of 1,2-cyclopentanedicarboxylic acid, which are related by symmetry. This phosphoryl group might interact with a Mn^{2+} ion at the active site, even more effectively than would a carboxyl group. In a possibly similar case, replacement of the carboxyl group of L-leucine by a phosphonyl group has been found to improve its binding affinity for pig kidney microsomal leucine aminopeptidase by a factor of 55 [compare results reported by Andersson et al. (Andersson, L.; Isley, T. C.; Wolfenden, R. *Biochemistry* **1982**, *21*, 4711) with those reported by Lejczak et al. (Lejczak, B.; Kafarski, P.; Zygmunt, J. *Biochemistry* **1989**, *28*, 3549)].

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The fact that an intermediate in glycolysis is an extremely potent inhibitor of this enzyme, although mechanistically reasonable, raises unexpected questions concerning the location and function of prolidase. Freeze-clamp studies have shown that the physiological concentration of P-enolpyruvate in whole rat kidney, as in other mammalian tissues, is approximately 5×10^{-5} M.[11] Thus, the physiological concentration of P-enolpyruvate is roughly 6000-fold higher than the K_i value that we observed for inhibition of prolidase. The concentration of enzyme in whole kidney, based on the purification procedure, is in the neighborhood of 10^{-7} M.[12] The K_m value observed for a typical substrate, Gly-Pro, is 1.7×10^{-3} M, so that extremely high substrate concentrations, well in excess of 1 M, would be needed to completely overcome the inhibitory effects of intracellular P-enolpyruvate.[13]

Earlier evidence suggests that prolidase is located in the cytoplasm,[4,5] but its existence at other locations does not appear to have been excluded. Recent work has identified another renal peptidase, which does not act on proline-containing dipeptides, as an ectoenzyme anchored to the cell surface through phosphatidylinositol.[14] In a similar location, prolidase would presumably escape inhibition by cytoplasmic P-enolpyruvate. The enzyme might then be susceptible to inhibition by ionized compounds approaching from the cell's exterior, simplifying the design of potent inhibitors that would be active in vivo. These possibilities remain to be explored.

Acknowledgment. We are grateful to the National Institutes of Health (Grants GM-18325 and AI-25697) for support of this work.

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(13) An externally introduced competitive inhibitor, even if it were bound as tightly as P-enolpyruvate, would need to be present at extremely high concentrations in the cytoplasm to produce any noticeable effect.
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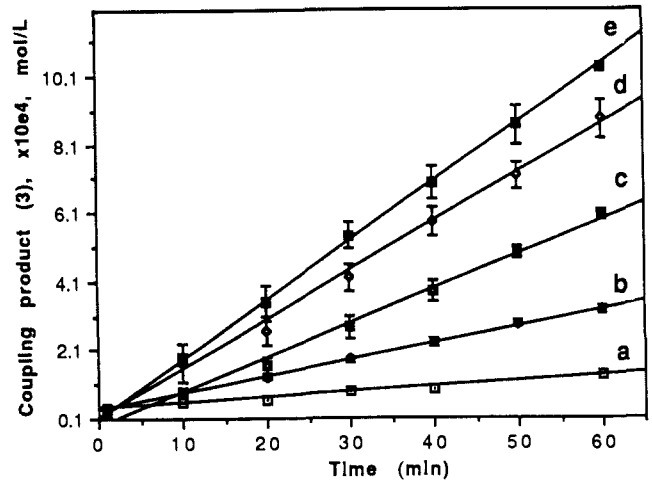
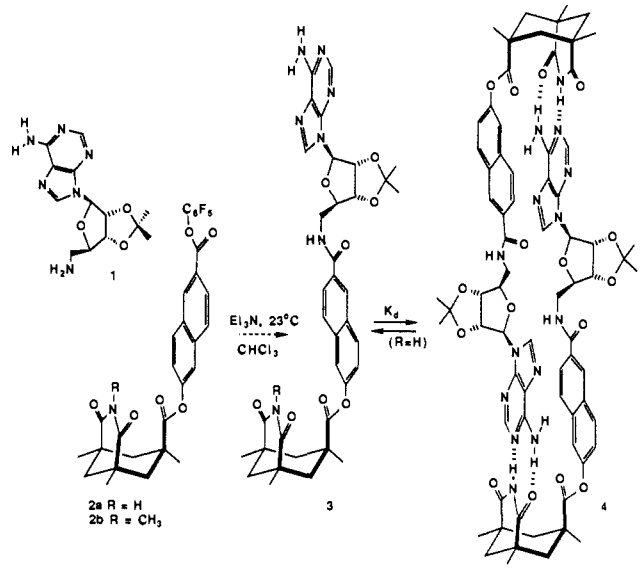


Figure 1. Initial rates of product formation (Scheme I). Plots of initial appearance of coupling products vs time as determined by HPLC. All reactions were performed with initial concentrations of [1] = [2] = 8.2 mM in $CHCl_3$ with 4 equiv of Et_3N added as a general base. Each run was independently performed in triplicate, and error bars represent standard deviations for each point. (a) Reaction of **1** and the N-methylated **2b**. (b) Reaction of **1** and **2a** with 1 equiv of 2,6-bis(acylamino)pyridine. (c) Reaction of **1** and **2a**. (d) Reaction of **1** and **2a** with 0.2 equiv of **3** added as autocatalyst. (e) As in d with 0.5 equiv of **3**.

Scheme I



A Self-Replicating System

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The ability of nucleic acids to act as templates for self-replication has been unique. In living systems, single strands act as templates during phosphate transfer reactions. In nonenzymatic systems, autocatalysis[1] can be observed during these reactions. Here we show that base pairing can also enhance acyl transfer reactions.

The reaction involves the coupling of the amino adenosine **1** to the pentafluorophenyl ester **2a** (Scheme I), and its shows the following features:

1. *The reaction exhibits autocatalysis.* Addition of the product **3** the reaction mixture results in an increase in the initial coupling rate (Figure 1, entries c-e).

2. *The product is self-complementary.* Dimerization occurs (**3** → **4**, Scheme I), and the value measured by NMR dilution studies[2] for the dimerization constant is $K_d = 630$ M^{-1}. This value is consistent with expectations involving base pairing and aryl stacking, with some attenuation by steric effects in the middle of the structure.[3]

3. *The reactions of* **2a** *proceed through the formation of base-paired complexes.* The imide **2a** reacts some 10 times faster than the *N*-methyl derivative **2b**, a factor that can hardly be attributed to classic steric effects at a site so remote from the reacting centers. Moreover, 2,6-bis(acylamino)pyridine acts as a competitive inhibitor by forming a nonproductive complex, **5** (Scheme II). Titration of **2a** with 5-*O*-acetyl-2′,3′-*O*-iso-

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