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'Irreversible' slow-onset inhibition of orotate phosphoribosyltransferase by an amidrazone phosphate transition-state mimic

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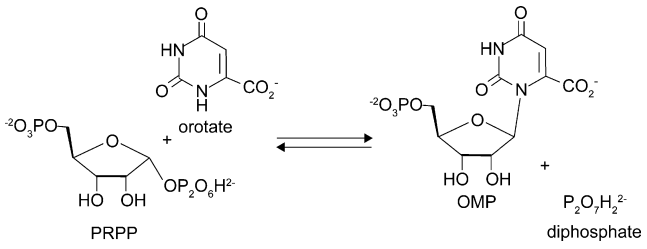
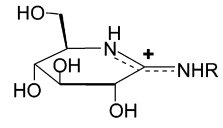
Abstract—A mimic of the putative transition-state intermediate has been synthesized and found to be a very slow-onset inhibitor of yeast orotate phosphoribosyltransferase. The mechanism of inhibition may involve a rate-determining isomerization of the enzyme to a form receptive to the inhibitor, which then remains tightly bound.

Orotate phosphoribosyltransferase (EC 2.4.2.10, OPRTase) catalyzes the formation of a carbon–nitrogen bond between the ribose moiety of α-D-5-phosphoribosyl 1-diphosphate (PRPP), and orotic acid (OA) to give orotidine 5′-phosphate (OMP), the precursor to all of the pyrimidine nucleotides.

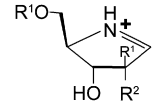


Interruption of pyrimidine nucleotide synthesis may offer a strategy for combating a variety of pathologies including cancer, parasitic diseases, and virus infections. Until now no tight-binding inhibitor of a pyrimidine PRTase has been reported.[1]

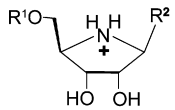
The chemical mechanism of OPRTase from *Salmonella typhimurium* has been shown to proceed through a transition state that resembles a classical oxocarbenium ion.[2] Similar transition-states are implicated in reactions mediated by nucleoside hydrolase,[3,4] purine nucleoside phosphorylase (PNPase),[5] hydrolytic glycosidases,[6–10] and HGPRTase.[11] Thus compounds that mimic an oxocarbenium in charge and/or structure might be expected to have significant affinity for these classes of enzymes, and function as potent inhibitors. Indeed, both naturally occurring and synthetic materials have been identified that bind exceptionally well to each of these enzymes. In particular, the 'alkaloidal sugars,' desoxynojirimycin and swainsonine with a positive charge on nitrogen at biological pH, are credible inhibitors of glucosidases and mannosidases, respectively, even though neither has the requisite planar geometry associated with a carbenium ion.[6,10]



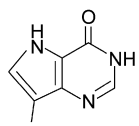
1, R = -H
2, R = -NH_2
3, R = -OH

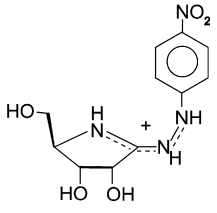


4, R^1 = -OH, R^2 = -H
5, R^1 = -H, R^2 = -OH



6, R^1 = -H, R^2 = -Ph
7, R^1 = -H, R^2 =
8, R^1 = -OPO_3H^-, R^2 =





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Keywords: Phosphoribosyltransferase; Slow-onset inactivation; Amidrazone; Oxocarbenium; Transition state.
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Even stronger binding to and inhibition of glycosidases has been effected with several synthetic amidines (**1**–**3**)[6] and imines **4**[7] and **5**[9] which mimic both the charge and geometry of the oxocarbenium transition state allowing these compounds to be characterized as true transition-state analogues.[6]

Two features that the transition states of nucleoside hydrolases, PNPases, and PRTases share are significant bond order between the entering/leaving nucleobase and the carbocationic ribose entity with little, if any, bonding associated with the attacking/departing nucleophile (water, P_i, or PP_i, respectively).[1] Thus it might be supposed that a potent transition-state inhibitor for these enzymes would necessitate the incorporation of a nucleobase analogue into the structure. This supposition has been borne out, for example, with the recent syntheses of compounds **6**–**9**. Compounds **6**, **7**, and **9** are potent inhibitors of nucleoside hydrolase and PNPase,[3–5] while **8** is a potent inhibitor of human and malarial HGPRTases.[11]

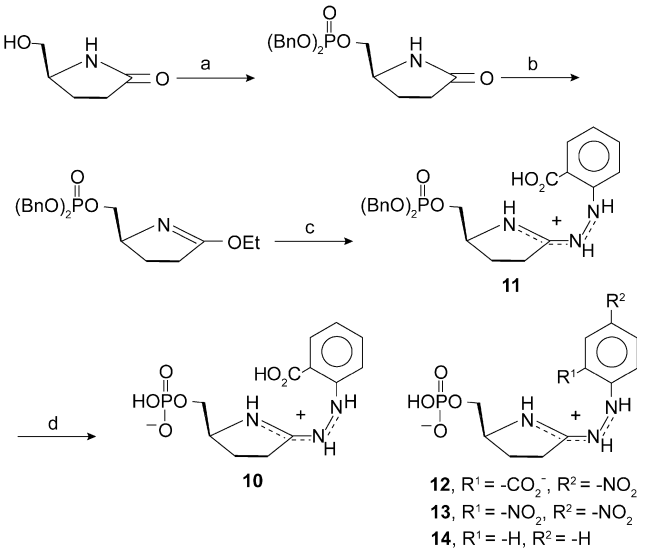
Crystal structures of OPRTase from yeast[12] and *Salmonella*[13] with OMP bound in the active site showed the 2′- and 3′-hydroxyls to be exposed to solvent water. Additionally, the fact that three PRPP mimics with rather diverse modifications at the 2′- and 3′ position(s) of PRPP were found to be reasonable inhibitors of OPRTase[14] indicated that the 2′- and 3′-hydroxyls might not be crucial to a transition-state analog for OPRTase.

Taken as a whole, these results led us to hypothesize that a compound such as **10**, incorporating sp^2 geometry and a plus charge at the anomeric position along with an analog of orotic acid, would act as a transition-state mimic for OPRTase.

This communication is an account of the synthesis of a potent slow-onset inhibitor, amidrazone **10** (Scheme 1) along with three other non-inhibitory amidrazones.

The synthesis of **10** began with a Mitsunobu phosphorylation[15] of (*S*)-(+)-5-hydroxymethyl-2-pyrrolidinone with dibenzyl phosphate mediated by triphenyl phosphine and diethyl azodicarboxylate to give the 5-dibenzyl phosphoryl pyrrolidinone in excellent yield.[16] The orotate 'mimic' was introduced via a two-step procedure involving the formation of an ethoxy imino ether at C-2 upon treatment of the phosphorylated pyrrolidinone with triethyloxonium hexafluorophosphate followed by reaction of the imino ether with 2-hydrazino benzoic acid in the presence of *N*,*N*-diisopropyl-*N*-ethylamine in CH_2Cl_2 to yield **11**. When **11** was treated with 8 M HCl at room temperature for several hours the desired amidrazone phosphate, **10**, was formed in excellent yield along with benzyl chloride. The phenyl, 4-nitro-2-carboxyl, and 2,4-dinitro amidrazones **12**–**14** were produced in identical fashion.

When yeast apo OPRTase[17] was incubated with amidrazone **10** at concentrations ranging from 0.05 to 2 mM at 25 °C a rather slow but steady diminution



Scheme 1. Synthesis of the amidrazone OPRTase inhibitor, **10**, and related molecules. Reagents and conditions: (a) $(BnO)_2PO_2H$, Ph_3P, DEAD, THF, 2 h, 25 °C; (b) $Et_3O^+PF_6^-$, CH_2Cl_2, 2 h, 25 °C; (c) *o*-hydrazinoPhCO$_2$H, *i*-Pr_2NEt, CH_2Cl_2, 16 h, 25 °C; dil aq HCl; (d) 8 M HCl, 16 h, 25 °C.

in enzyme activity was observed during the course of over 20 days (Fig. 1) when compared with a control (same enzyme incubated without inhibitor).[18] Identical rates of inactivation were observed for either of the rather stable 1:1 complexes[19] of OPRTase with substrates, OMP and PRPP. On the other hand, incubation of OPRTase with **10** in the presence of excess (1, 5, or 10 mM) OMP afforded significant protection of the enzyme against inactivation (Fig. 2), an indication that inactivation is occurring at the active site of the enzyme.

No inactivation whatsoever could be detected with compounds **12**–**14**.[20] Thus, it appears that the pendant *ortho*-carboxyl group of **10** is required for recognition by OPRTase, in accordance with the observation that neither uracil nor UMP binds to OPRTase.[21] The observed rates of inactivation were found to be pseudo-first order in enzyme (Fig. 1). Additionally, the rate of inactivation as a hyperbolic function of [**10**] was not as one would observe for a simple first-order process with respect to concentration of inhibitor (Fig. 3). The onset of inhibition is, however, consistent with the mechanism depicted in Scheme 2, wherein the most common conformation of the enzyme is in equilibrium with a second minor form, the latter allowing the binding of **10** to form an inactive complex.

Non-linear regression analysis of the data yielded $k_1 = 0.0157\ h^{-1}$ (no $MgPP_i$) and $0.006\ h^{-1}$ (presence of $MgPP_i$). Regression analysis cannot yield independent values for k_{-1} and k_2 but limits on them can be placed.[22] At [**10**] > 0.6 mM, k_1 becomes the rate-limiting step in inactivation of OPRTase. The rates of inactivation observed are unusually small when compared to those tabulated by Schloss.[23] The presence of $MgPP_i$ caused

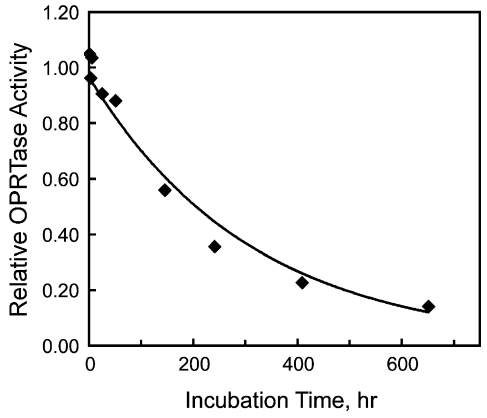


Figure 1. Time-dependent inactivation of OPRTase by 2 mM amidrazone phosphate compound **10** in the presence of 2 mM PP_i plus 3 mM Mg^{2+} at 20 °C. Values are corrected by controls incubated the same amount of time without inhibitor. Fitted line corresponds to pseudo-first-order decay with $k_{obsd} = 0.0040\ h^{-1}$.

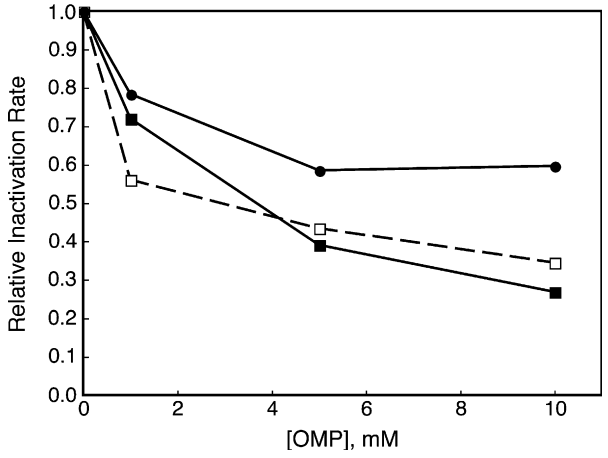


Figure 2. Relative rates of inactivation of yeast apo OPRTase as a function of the concentration of OMP at various levels of amidrazone **10** (—■—, 0.5 mM; --□--, 1.0 mM; —●—, 2.0 mM) at 20 °C. Values are expressed as the ratio of the pseudo-first-order rate constants for inactivation by **10** in the presence and absence of OMP, respectively.

a 2.5-fold decrease in the rate of inactivation, perhaps indicating that both **10** and PP_i compete, at least partially, for a binding site on the enzyme.

In an attempt to determine the rate of dissociation of the enzyme–inhibitor complex, excess **10** was removed by dialysis. Aliquots of this material were then incubated with the assay mixture (without OMP) and activity determined at intervals during 16 days. No regain of activity could be detected (data not shown). Thus the inactivation is essentially irreversible ($k_{-2} \approx 0$). Such apparent irreversibility is not without precedent. Indeed the half-life for the release of 2-carboxy-D-arabinitol 1,5-bisphosphate from spinach ribulose bisphosphate carboxylase[24] is reported to be 530 days while the value accompanying release of L-methionine sulfoximine *N*-phosphate from *Escherichia coli* glutamine synthetase[25] was estimated to be of the order of 10^5–10^6 years at biological pH 7. Slow-onset tight binding inactivation is often associated with compounds that have features in common with the transition-state of the enzyme-catalyzed reaction.[23,26]

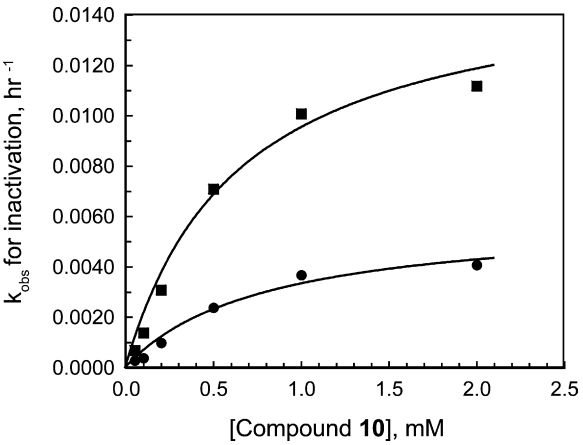
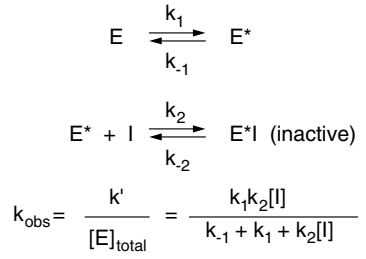


Figure 3. Rate of inactivation (as k_{obsd}) of yeast apo OPRTase as a function of the concentration of amidrazone **10** at 20 °C. The upper curve (squares) yields $k_1 = 0.0157\ h^{-1}$. The lower curve (circles) was obtained in the presence of 3 mM Mg^{2+} plus 2 mM PP_i and yielded $k_1 = 0.006\ h^{-1}$. Values of k_{-1} and k_2 were chosen as described.[23]



Scheme 2. Model (with derived kinetic expression) for inactivation after slow isomerization of enzyme. The k' is the true rate of inactivation and k_{obsd} is the measured pseudo-first-order rate constant. E represents OPRTase in its high-probability conformation, E* is the OPRTase form that can bind the amidrazone inhibitor, I.

It is tempting to conclude that the values of k_1 relate to the rate at which OPRTase changes its conformation sufficiently to accept the inhibitor and that this process correlates with the opening/closing of a catalytically important motional loop—a common feature of PRTases in general.[27] Indeed Wang et al. have characterized such loop dynamics in the *Salmonella* OPRTase.[28] However our higher k_1 value is still only about 1% of the rate deduced from proteolysis[28] experiments and many orders of magnitude slower than loop motions measured by NMR[28] suggesting that much larger conformational changes may be required to accommodate the binding of **10**. Finally, it is possible that incorporation of *cis*-2′,3′-hydroxyls into **10** might improve the inhibitory properties of **10**, in particular the rate at which the enzyme binds to the more exact inhibitor. Efforts are underway to extend our synthetic methodology to include that feature.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2006.08.109.

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$$k_2 = \frac{k_{-1} + k_1}{\Phi}$$

where Φ is the concentration of inhibitor and where $k_{obsd} = k_1/2$. For example, in the case without $MgPP_i$ present, $\Phi = 0.65$ mM and the minimal value (i.e., when $k_{-1} \approx 0$) of k_2 is 0.025 h^{-1}. Thus at [**10**] ≈ 0.6 mM and k_2 [**10**] $\geqslant k_1$.
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