# Synthesis and Enzymic Evaluation of 4-Mercapto-6-oxo-1,4-azaphosphinane-2-carboxylic Acid 4-Oxide as an Inhibitor of Mammalian Dihydroorotase

Michael K. Manthey,* Danny T. C. Huang, William A. Bubb, and Richard I. Christopherson

*Department of Biochemistry, University of Sydney, Sydney, NSW 2006, Australia*

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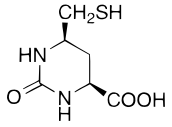
The design, synthesis, and enzymic evaluation of *cis*- and *trans*-4-mercapto-6-oxo-1,4-azaphosphinane-2-carboxylic acid 4-oxide **5** against mammalian dihydroorotase is presented. The design strategy for **5** was based on the strong affinity of phosphinothioic acids for zinc and that **5** also resembles the postulated tetrahedral transition state for the enzyme-catalyzed reaction. The synthesis of **5** utilized a novel protection/deprotection sequence upon 4-hydroxy-6-oxo-1,4-azaphosphinane-2-carboxylic acid 4-oxide **4**, followed by incorporation of α-phenyl benzenemethanethiol and exhaustive deprotection to afford **5** in 40% overall yield from **4**. The activities of both isomers of **5** as inhibitors of mammalian dihydroorotase were marginally greater than that of the parent phosphinic acid **4**, indicating a weak binding enhancement due to the phosphinothioic acid moiety.

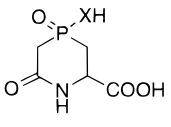
## Introduction

Dihydroorotase (DHOase) catalyzes the third reaction of the pathway for de novo biosynthesis of pyrimidine nucleotides, the reversible cyclization of *N*-carbamyl-L-aspartate (CA-asp) **1** to L-dihydroorotate **2** (Figure 1). A zinc atom bound at the active site of the enzyme has been implicated in the catalytic mechanism.[1] It has been proposed that the mechanism is similar to that of a zinc protease in which the zinc atom stabilizes the tetrahedral transition state of the reaction by formation of a coordination complex to two oxygen atoms at C-6 of the dihydropyrimidine ring (Figure 1).[2]

Inhibitors of DHOase have potential use as anticancer[3] or antimalarial drugs.[4] Several inhibitors of DHOase have been designed as chelators of the active site zinc atom.[5−7] To date these have principally relied on the strong affinity of sulfur for zinc, with the most potent inhibitor, the *cis*-mercaptomethyl analogue **3**,




**3**




**4** X = O
**5** X = S

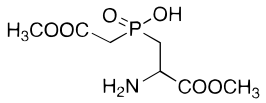
exhibiting an apparent $K_i$ of 140 nM at pH 7.4 ($K_m$ of **2** is 19 $\mu$M at pH 7.4). However **3** only has a relatively short half-life in vitro, thereby making its clinical use limited.

A number of transition-state analogues based on sulfone, sulfoxide,[8] carboxylate,[9] and phosphinate[10] groups have also been developed. One of these, the phosphinic acid **4**, was designed as a stable transition-state analogue which closely resembles the tetrahedral geometry of the transition state while at the same time being relatively nonimposing with respect to steric constraints.[11]

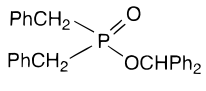
An enzyme inhibitor incorporating multiple structural attributes, which individually are known to promote binding, would be expected to have a greater probability of yielding a potent inhibitor of DHOase. Consequently the phosphinothioic acid **5**, which not only mimics the transition state of the enzyme-catalyzed reaction but may also provide a strong chelation effect via a strong S−Zn interaction,[11] was designed as a transition-state inhibitor for DHOase. We report herein the synthesis and enzymic evaluation of *cis* and *trans*-**5** as inhibitors against mammalian DHOase.

## Chemistry

The synthetic strategy employed for the phosphinothioic acids **5a**,**b** is outlined in Scheme 1. The starting material for the synthesis, the parent phosphinic acid **4**, has previously been synthesized by the cyclization of the amino acid **6** with 1 equiv of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (DEPC) at pH 5.0 in a yield of 21%.[10] We have increased the yield of **4** in the cyclization reaction to 66% by raising the pH of the reaction medium to 5.6 and by employing a 1-fold excess of DEPC. Selective protection of the carboxylic acid moiety in **4** was required prior to functionalization of the phosphinic acid. A direct protection strategy proved troublesome. For example, esterification of **4** with $CH_3OH/H^+$ under mild conditions resulted in extensive ring opening to yield the amino acid ester **7**. Consequently, an indirect approach




**7**




**10**

was employed for the selective protection of the carboxylic acid in **4**. The diphenylmethyl group was selected to protect both the carboxylic and phosphinic acid groups in **4** to give a UV-active product that could be purified chromatographically and easily deprotected under mild conditions at the end of the synthesis.

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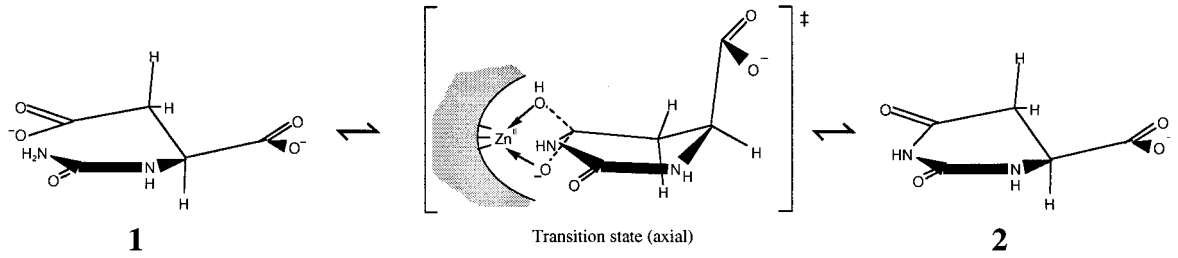
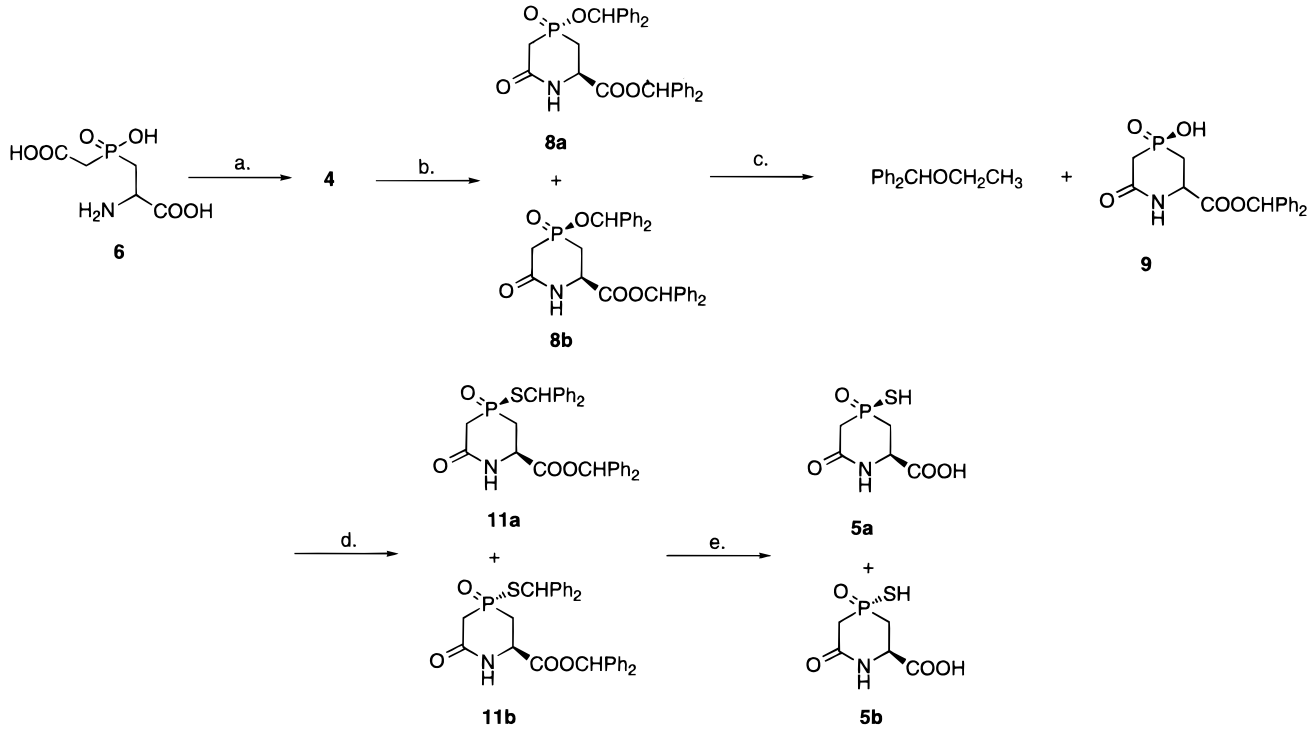



**Figure 1.** The reaction catalyzed by dihydroorotase.

**Scheme 1**[a]




[a] Reagents: (a) 1-(3-Dimethylaminopropyl)-3-ethylcarbodimide, pH 5.6, 45−60 °C; (b) $Ph_2CN_2$; (c) $CH_3Ch_2OH$; (d) $Ph_2CHSH$, DECP, $Et_3N$; (e) TFA.

Reaction of **4** with a slight excess of diphenyldiazomethane yielded the diasteromeric bisdiphenylmethyl esters **8a** and **8b** which were readily separable by column chromatography.

The configurations of **8a** and **8b** were established by assignment of their $^1H$ NMR spectra, with the aid of spin-decoupling to clarify relationships characterized by relatively small *J* values, together with nuclear Overhauser effect spectrometry (NOESY) experiments. For the latter, application of a weak pulsed field gradient during the mixing time[12] provided a significant improvement in spectral quality which permitted observation of the relatively small enhancements which were critical to establishing the stereochemistry. NMR data for the pair of isomers, **8a** and **8b**, and their thio analogues, **11a** and **11b**, are presented in Table 1. The data support assignment of the relationship between the substituents bearing the dibenzyl groups as trans in **8a** and **11a** but cis in **8b** and **11b**.

Thus in both **8a** and **8b**, H-2 has the axial configuration as evidenced by the relative magnitudes of the vicinal coupling constants to H-3 and H-3′. Consequently, the bulky diphenylmethylcarboxylate moiety at C-2 must have the equatorial configuration in both isomers. In addition, **8a** has a clearly resolved coupling between the cis proton at C-3 and one of the protons attached to C-5. Given the constraints already noted for the bulky substituent at C-2, the requisite **W** geometry for coupling across four single bonds[14] fixes the conformation of **8a** and the assignments therein of H-3 and H-5. The stereochemistry of the substituents attached to the phosphorus atom follows from observation of a weak nuclear Overhauser effect (NOE) between the benzyl proton attached to the phosphinate group, designated H-A, and both H-3 and H-5 (Figure 2). There was some sharpening of the H-3 resonance upon spin-decoupling of H-1, indicating a quasi-**W** pathway beween each of these protons, consistent with the conformation assigned.

Vicinal coupling constants between H-2 and H-3/H-3′ in **8b** are of similar magnitude to those in **8a** but there was only a very weak additional coupling (evidenced by resonance sharpening on spin-decoupling) between H-3′ and the lower frequency resonance of the H-5/H-5′ pair. Rather, a clearly resolved 1.2 Hz splitting in the resonance of the same C-5 proton was shown to be associated with coupling to the NH proton, designated H-1. Further, the C-5 resonance involved in this putative **W**-coupling and the C-3 proton with the larger vicinal coupling show NOE cross-peaks to H-A. These

**Table 1.** $^1$H NMR Data for Compounds Used for Stereochemical Assignments[a]

Chemical Shifts

| compd | $H_1$ | $H_2$ | $H_3$ | $H_{3'}$ | $H_5$ | $H_{5'}$ | $H_A$ | $H_B$ | $H_{arom}$ |
|---|---|---|---|---|---|---|---|---|---|
| **8a** | 6.39 | 4.07 | 2.21 | 1.95 | 2.72 | 2.78 | 6.55 | 6.80 | 7.06−7.36 |
| **8b** | 6.42 | 4.39 | 2.41 | 1.90 | 2.77 | 2.59 | 6.48 | 6.80 | 7.08−7.35 |
| **11a** | 6.44 | 4.12 | 2.20 | 2.11 | 2.83 | 3.01 | 5.94 | 6.89 | 7.06−7.54 |
| **11b** | 6.49 | 4.49 | 2.56 | 1.92 | 2.93 | 2.56 | 5.89 | 6.89 | 7.14−7.50 |

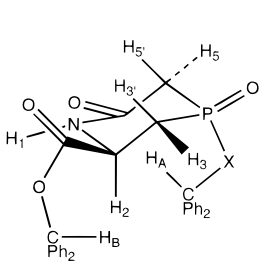
Coupling Constants

| compd | $J_{1,2}$ | $J_{1,5'}$ | $J_{2,3}$ | $J_{2,3'}$ | $J_{2,P}$ | $J_{3,3'}$ | $J_{3,5}$ | $J_{3,P}$ | $J_{3',P}$ | $J_{5,5'}$ | $J_{5,P}$ | $J_{5',P}$ | $J_{A,P}$ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **8a** | 1.9 | | 3.5 | 11.8 | 10.7 | 15.0 | 1.5 | 20.1 | 9.8 | 17.4 | 18.4 | 14.8 | 9.2 |
| **8b** | 2.7 | 1.2 | 3.7 | 11.8 | 12.7 | 14.8 | | 17.7 | 13.5 | 16.5 | 17.4 | 16.8 | 8.8 |
| **11a** | 2.0 | | 3.4 | 11.9 | 11.8 | 14.8 | 1.3 | 15.3 | 11.8 | 17.4 | 12.8 | 17.2 | 9.4 |
| **11b** | 2.7 | 1.2 | 3.4 | 11.9 | 12.7 | 15.0 | | 18.3 | 6.1 | 16.5 | 18.7 | 11.9 | 9.2 |

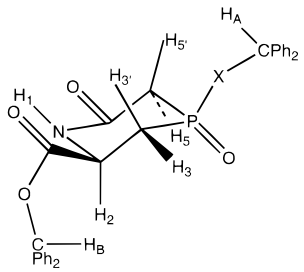
Observed NOE

| compd | int[b] | $H_1$ | $H_2$ | $H_3$ | $H_{3'}$ | $H_5$ | $H_{5'}$ | $H_A$ | $H_B$ |
|---|---|---|---|---|---|---|---|---|---|
| **8a** | s | $H_2$ | $H_1$, $H_3$ | $H_2$, $H_{3'}$ | $H_3$ | $H_{5'}$ | $H_5$ | | |
| | m | | $H_5$ | | | $H_2$ | | | |
| | w | $H_5$, $H_{5'}$ | $H_{3'}$, $H_B$ | $H_A$ | $H_2$, $H_{5'}$ | $H_1$, $H_A$ | $H_1$, $H_{3'}$ | $H_3$, $H_5$ | $H_2$ |
| **8b** | s | $H_2$ | $H_1$, $H_3$, $H_5$ | $H_2$, $H_{3'}$ | $H_3$ | $H_2$, $H_{5'}$ | $H_5$ | | |
| | m | | | | | | | | |
| | w | $H_{3'}$ | $H_{3'}$, $H_B$ | | $H_1$, $H_2$, $H_A$ | | $H_A$ | $H_{3'}$, $H_{5'}$ | $H_2$ |
| **11a** | s | $H_2$ | $H_1$, $H_3$ | $H_2$, $H_{3'}$ | $H_3$ | $H_{5'}$ | $H_5$ | | |
| | m | | $H_5$ | | | $H_2$ | | | |
| | w | | $H_{3'}$ | $H_A$ | $H_2$ | | | $H_3$ | |
| **11b** | s | $H_2$ | $H_3$, $H_5$ | $H_2$, $H_{3'}$ | $H_3$ | $H_2$, $H_{5'}$ | $H_5$ | | |
| | m | | $H_1$ | | | | | | |
| | w | $H_{3'}$ | $H_B$ | | $H_1$, $H_2$ | | $H_A$ | $H_{5'}$ | $H_2$ |

[a] Chemical shifts and coupling constants were determined by first-order analysis for all spin systems where $\Delta\nu/J > 10$ and otherwise by ABX calculation[13] following simplification of the spectra by spin-decoupling. [b] Relative intensity of NOE cross-peak: s, strong; m, medium; w, weak.



**8a** **11a**



**8b** X=O
**11b** X=S

data can be rationalized by the structure depicted for **8b**. The 1,3-diaxial interaction between the P═O and C═O bonds, which would accompany the change in stereochemistry of the phosphorus substituents in a full chair conformation, is evidently sufficient to cause a conformational change to a half-chair. This conformational change removes the **W**-pathway between H-3 and H-5 which is evident in **8a**. The observation of a weak unresolved coupling between H-3′ and H-5′ may reflect some population of a boat conformation; further evidence for conformational mobility in both **8a** and **8b** is seen in the observation of weak NOE enhancements between H-2 and H-3′. The majority of observed enhancements are, however, entirely consistent with the given conformation for each isomer.

The combined yield of **8a** and **8b** was dependent upon the solvent employed in the alkylation. Use of DMF as solvent resulted in a 76% yield of **8** (**8a**:**8b,** 3:2) whereas ethanol led to a marked reduction to a 44% combined yield. The solvent dependence was investigated further by subjecting **8a** to incubation in ethanol at reflux. After 1 h of reflux, **8a** had been completely consumed and diphenylmethylethyl ether and the phosphinic acid **9** were formed exclusively. Reflux of **8b** under the same conditions also resulted in the formation of **9** and diphenylmethylethyl ether. To investigate the general applicability of the reaction, the diphenylmethyl phosphinate **10** was synthesized and incubated in ethanol at reflux. No reaction was evident after 1 h with unreacted **10** being recovered. Hence it would seem that de-esterification of **8** in ethanol is a consequence of specific steric and/or electronic factors. We tentatively propose that ethanol serves as a general acid/base catalyst in the deprotonation of the acidic H-5ax proton in **8**, with loss of the relatively stable diphenylmethyl cation, which is captured by ethanol driving the reaction. (The H-2 protons in **4** are gradually exchanged with deuterium in D2O solution, indicating their acidic nature.) A postulated reaction mechanism for the deprotection of **8a** is shown in Figure 3. A similar reaction mechanism with a chair-like transition state can be drawn for **8b** after a conformational change which brings the diphenylmethyl phosphinate ester into the equatorial configuration.

Syntheses of phosphinothioic esters from phosphinic acids have generally been carried out via the intermediacy of phosphinyl chlorides generated by the action of either thionyl chloride or phosphorus pentachloride on the phosphinic acid.[15] These classical methods would be unsuitable in the present case due to the concomitant formation of HCl which would rapidly decompose **8**. Several thioesterification methods were tested on the dibenzylphosphinic acid **12** which served as a model compound. For example, incubation of **12** with thionyl chloride or phosphorus pentachloride, in the presence of triethylamine to scavenge HCl, followed by reaction with α-phenyl benzenemethanethiol failed to yield any

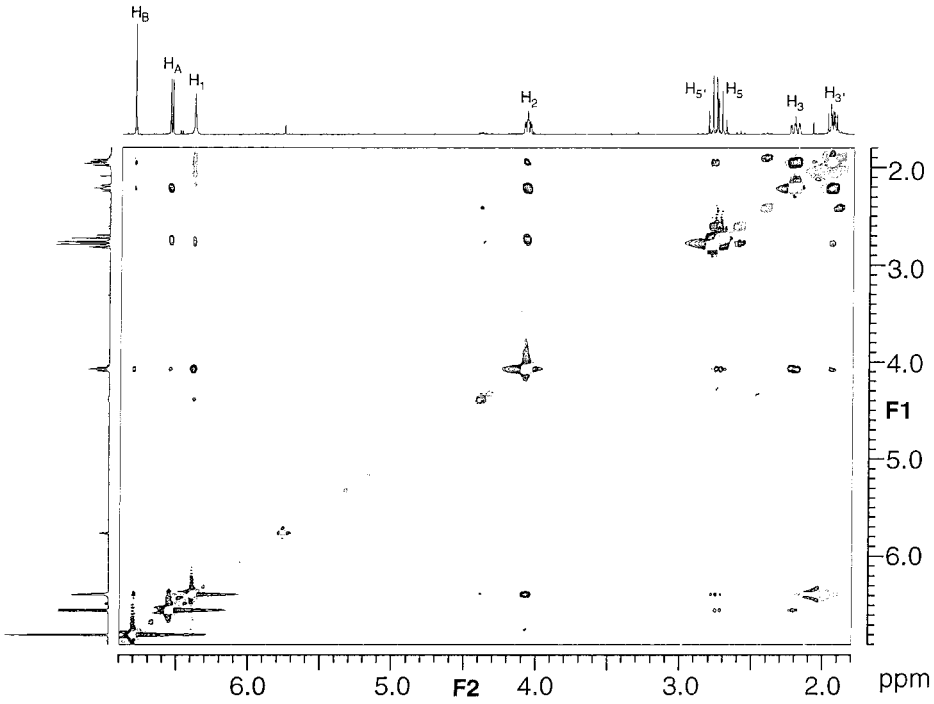



**Figure 2.** Part of a NOESY spectrum obtained for the bis(diphenylmethyl) ester **8a**. NOE cross-peaks are shown in darker shading compared with the diagonal and a cross-peak due to exchange between the NH proton and $H_2O$ contained in the solvent. For details, see the Experimental Section.

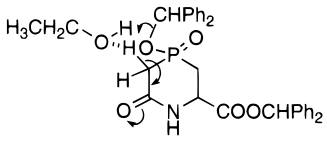
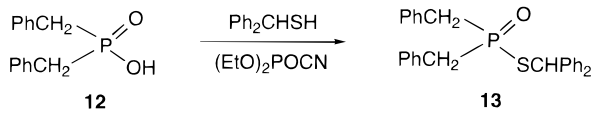



**Figure 3.** Proposed reaction mechanism for the ethanol-induced decomposition of **8a**.




phosphinothioate. Ultimately, diethylcyanophosphonate was found to be an efficient coupling agent for the synthesis of hindered phosphinothioic esters from **12**. In this manner, the diphenylmethylthio ester **13** was prepared in 56% yield from the reaction of **12** and α-phenyl benzenemethanethiol in the presence of triethylamine. Extension of this reaction to **9** resulted in the formation of the diastereomeric esters **11a**,**b** in 33% and 31% yields, respectively, after purification by selective crystallization. Attempted purification of **11** via column chromatography on silica or alumina (acidic, basic, or neutral) led to virtually complete decomposition (90−100%) of the individual isomers. Interestingly, neither **11a** nor **11b** underwent extensive decomposition in boiling ethanol. Presumably this is a consequence of the increased P−S bond length in **11** compared to the corresponding P−O bond length in **8** which does not allow a favorable transition-state geometry to be attained.

The configurations of **11a** and **11b** were determined by analysis of their NMR spectra. The *J* coupling patterns and NOE enhancements for the respective isomers (Table 1) are entirely analogous to those observed for **8a** and **8b**, with the notable exception that the relative intensities of weaker NOE cross-peaks were even further attenuated in the thio compounds.

Removal of the diphenylmethyl protecting groups from **11** was readily achieved employing trifluoroacetic acid in the presence of anisole as cation scavenger. In this manner, the phosphinothioic acids **5a** and **5b** were prepared in high yield from **11a** and **11b,** respectively.

## Enzymic Evaluation

The hamster dihydroorotase domain was overexpressed in the *Escherichia coli* strain SØ1263/pCW25 and purified as previously described.[6] Compounds **5a** and **5b** were tested for their ability to inhibit the catalytic activity of hamster DHOase. Dixon plots for inhibition of DHOase by **5a** and **5b** (Figure 4) showed that both isomers were potent inhibitors of the enzyme with inhibition constants ($K_i$ values) of 2.9 ± 0.6 and 3.1 ± 0.3 $\mu$M, respectively, at pH 7.4 for hydrolysis of L-dihydroorotate to *N*-carbamyl-L-aspartate. The inhibitory activities of **5a** and **5b** were comparable to that of the parent phosphinic acid **4** ($K_i$ = 4.0 ± 1.0 $\mu$M, Figure 4). To investigate whether DHOase was rapidly converting **5** into **4**, thereby giving rise to the similar $K_i$ values, the isomers **5a** and **5b** were incubated in phosphate-buffered $D_2O$ (pH 7.2) at 38 °C with DHOase and the reaction was monitored by $^1$H NMR over a 15 min period corresponding to the time period of the assay. No turnover of **5** to **4** was observed. In fact **5a** and **5b** were stable under the above conditions over a period of several weeks with no hydrolysis observable over this time period by $^1$H NMR.

In conclusion, the phosphinothioic acids **5a** and **5b** have been synthesized and were tested as inhibitors of mammalian DHOase in vitro. Although both isomers were potent inhibitors of DHOase, replacement of a phosphinc acid with a phosphinothioic acid offered marginal enhancement of binding capability.

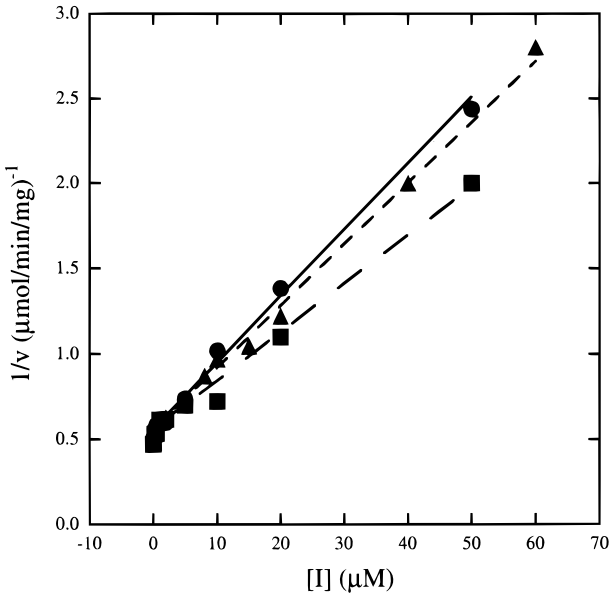



**Figure 4.** Dixon plots for inhibition of dihydroorotase by **4** (—■—), **5a** (—●—), and **5b** (-▲-). The DHOase activity was measured at fixed dihydroorotate concentration (20 $\mu$M) and variable inhibitor concentrations (0−60 $\mu$M) as described in Experimental Section. The data were fitted to eq 1. The initial velocities are in units of $\mu$mol of *N*-carbamyl-L-aspartate/min/mg of protein.

## Experimental Section

**Chemistry.** Melting points were determined thermoelectrically on a Reichert hot stage apparatus and are uncorrected. Infrared spectra were recorded on a Perkin-Elmer infrared spectrophotometer model 783 as mulls in Nujol and are reported in reciprocal centimeters ($cm^{-1}$). The $^1H$ NMR spectra were acquired on either a Bruker AC-200 or an AMX-600 spectrometer operating at 200 and 600 MHz, respectively. The spectra were measured in $CDCl_3$ unless otherwise stated. Each signal is described in terms of chemical shift as parts per million (ppm) relative to tetramethylsilane as the internal standard ($\delta$ 0.00). Signals are described as s (singlet), d (doublet), t (triplet), q (quartet), and m (multiplet). NOESY spectra were acquired with application of a weak pulsed field gradient during the mixing time of 1.2 s and processed with 5 Hz Gaussian enhancement in each dimension. Microanalyses (C, H, N) were performed by the Australian National University Analytical Services, Canberra.

Reactions were followed by TLC on Merck F254 silica gel plates. Merck silica gel (70−230 mesh) was used for column chromatography. All solvents and reagents were obtained from commercial sources and purified before use as required.

**(±)-4-Hydroxy-6-oxo-1,4-azaphosphinane-2-carboxylic Acid 4-Oxide, 4.** 3-[(Carboxymethyl)hydroxyphosphoryl]-alanine **6** (10 g, 48.0 mmol) and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (16 g, 83.5 mmol) were dissolved in water (500 mL), and the pH was adjusted to 5.6 with 1 M NaOH. The mixture was stirred at 50 °C for 24 h and then applied to a cation-exchange resin (AG 50W−X8) and eluted with water. The fractions having a pH between 0.5 and 2.0 were combined and evaporated to a small volume to afford **4** (6.04 g, 66%) as a white solid, mp 214−216 °C (lit.[10] mp 213 °C).

**(±)-*cis*- and *trans*-Diphenylmethyl 4-Diphenylmethoxy-6-oxo-1,4-azaphosphinane-2-carboxylate 4-Oxide, 8a,b.** To a solution of **4** (300 mg, 1.55 mmol) in dry DMF (10 mL) was added a solution of diphenyldiazomethane[16] in ether until the distinctive purple/red color of diphenyldiazomethane persisted for 1 h. Solvent was removed under vacuum and the residue purified via column chromatography, eluting with ethyl acetate/hexane (1:1 to 2:1) to afford **8a** (370 mg, 45%) followed by **8b** (248 mg, 31%) as two well-resolved diastereoisomers.

Data for **8a**: mp 175−176 °C (ethyl acetate/hexane). IR: 698, 745, 978, 1006, 1187, 1253, 1672, 1738, 3192 $cm^{-1}$. Anal. ($C_{31}H_{28}NO_5P$) C, H, N.

Data for **8b**: mp 159−160 °C (ethyl acetate/hexane); IR: 698, 743, 990, 1208, 1674, 1742, 3207 $cm^{-1}$. Anal. ($C_{31}H_{28}NO_5P$) C, H, N.

**Diphenylmethyl 4-Hydroxy-6-oxo-1,4-azaphosphinane-2-carboxylate 4-Oxide, 9.** A mixture of either **8a** or **8b** (200 mg, 0.38 mmol) in ethanol (3−5 mL) was refluxed for 0.5 h. Solvent was removed under vacuum, and the oily residue was redissolved in ethyl acetate (5 mL) and treated dropwise with stirring with ether to precipitate a white oily product. Solvent was decanted and the residue washed with ether to afford **9** (126 mg, 92%). After isolation as such, **9** was used directly for the preparation of **10**; analytically pure **9** was obtained by recrystallization from chloroform, mp 141−142 °C. $^1H$ NMR (ref $CD_2HSOCD_3 = \delta$ 2.50): $\delta$ 8.29 (s, 1, OH), 7.92 (d, 1, NH, $J$ = 4.3, 1.4 Hz), 7.27−7.46 (m, 10, ArH), 6.82 (s, 1, CH), 4.54 (dddd, 1, CH, $J$ = 4.3, 7.4, 7.5, 21.9 Hz), 2.71 (ddd, 1, $CH_2$, $J$ = 1.2, 16.2, 17.0 Hz), 2.60 (dd, 1, $CH_2$, $J$ = 16.2, 16.5 Hz), 2.33 (dddd, 1, $CH_2$, $J$ = 1.2, 7.4, 15.0, 15.4 Hz), 2.22 (ddd, 1, $CH_2$, $J$ = 7.5, 14.8, 15.4 Hz). IR: 699, 758, 976, 1120, 1233, 1683, 1734, 3203 $cm^{-1}$. Anal. ($C_{18}H_{18}NO_5P$) C, H, N.

The ethyl acetate/ether supernatant contained diphenylmethylethyl ether which could be purified via column chromatography eluting with hexane/EtOAc (95:5) to afford 49 mg, 61% of the ether as a thick oil. $^1H$ NMR: $\delta$ 7.15−7.35 (m, 10, ArH), 6.88 (s, 1, CH), 3.52 (q, 2, $CH_2$, $J$ = 7.3 Hz), 1.25 (t, 3, $CH_3$, $J$ = 7.3 Hz).

**(+)-*cis*- and *trans*-Diphenylmethyl 4-Diphenylmethylmercapto-6-oxo-1,4-azaphosphinane-2-carboxylate 4-Oxide, 11a,b.** To a suspension of **9** (200 mg, 0.56 mmol) in dichloromethane (5 mL) under an atmosphere of nitrogen were added triethylamine (62 mg, 0.62 mmol) and α-phenyl benzenemethanethiol[17] (116 mg, 0.58 mmol) followed by diethylcyanophosphonate (95 mg, 0.58 mmol). The mixture was stirred at room temperature for 24 h, diluted with dichloromethane (20 mL), and washed with water (2 × 20 mL). The organic phase was dried (sodium sulfate) and solvent removed under vacuum to afford a light yellow semisolid. This was swirled with ethyl acetate (10 mL) and allowed to stand at room temperature for 4 h. The precipitate was collected by filtration to afford essentially pure **11b** (93 mg, 31%). The filtrate was treated with hexane (10 mL) and left at 0 °C overnight. The precipitated material was collected and crystallized from ethyl acetate/diethyl ether to afford **11a** (99 mg, 33%).

Data for **11a**: mp 174−176 °C. IR: 1159, 1707, 1755, 3246 $cm^{-1}$. Anal. ($C_{31}H_{28}NO_4PS$) C, H, N.

Data for **11b**: mp 204−205 °C (chloroform/ethyl acetate). IR: 1165, 1682, 1750, 3223 $cm^{-1}$. Anal. ($C_{31}H_{28}NO_4PS$) C, H, N.

**(+)-*cis*- and *trans*-4-Mercapto-6-oxo-1,4-azaphosphinane-2-carboxylate 4-Oxide, 5a,b.** To a solution of **11a** or **11b** (100 mg, 0.18 mmol) in dichloromethane (1 mL) was added anisole (100 $\mu$L) followed by trifluoroacetic acid (2 mL). The mixture was stirred at room temperature for 2 h, solvent was removed under vacuum, and the residue was treated with ether (3 mL). The small amount of insoluble material was removed by filtration and the filtrate treated with hexane until cloudiness ensued. After the mixture stood at room temperature overnight, the resulting crystals of **5a** or **5b** respectively were collected and washed with a little diethyl ether/hexane (1:1).

Data for **5a**: yield 32 mg (81%); mp 173−176 °C. $^1H$ NMR (DMSO-$d_6$): $\delta$ 7.68 (dd, 1, NH, $J$ = 1.5, 3.4 Hz), 4.36 (dddd, 1, CH, $J$ = 3.4, 5.6, 8.6, 19.6 Hz), 3.05 (dd, 1, CH, $J$ = 16.1, 16.7 Hz), 3.04 (dd, 1, CH, $J$ = 12.9, 16.1 Hz), 2.56 (ddd, 1, CH, $J$ = 8.6, 14.7, 15 Hz), 2.49 (ddd, 1, CH, $J$ = 5.6, 11, 14.7 Hz). IR: 963, 1264, 1651, 1724, 3253 $cm^{-1}$. Anal. ($C_5H_8NO_4PS$) C, H, N.

Data for **5b**: yield 28 mg (72%); mp 169−172 °C. $^1H$ NMR (DMSO-$d_6$): $\delta$ 7.76 (d, 1, NH, $J$ = 3.8 Hz), 4.34 (dddd, 1, CH, $J$ = 3.8, 5.9, 8.0, 19.5 Hz), 3.02 (ddd, 1, CH, $J$ = 1.6, 14.8, 16.2

Hz), 2.96 (dd, 1, CH, $J$ = 11.2, 16.2 Hz), 2.65 (dddd, 1, CH, $J$ = 1.6, 5.9, 14.9, 17.0 Hz), 2.41 (ddd, 1, CH, $J$ = 7.8, 8.0, 14.9 Hz). IR: 925, 1243, 1613, 1724, 3224 $cm^{-1}$. Anal. ($C_5H_8NO_4PS$) C, H, N.

**Diphenylmethyl Dibenzylphosphinate, 10.** To a suspension of **13**[18] (500 mg, 2.03 mmol) in benzene (20 mL) at 4 °C under an atmosphere of nitrogen was added thionyl chloride (254 mg, 2.13 mmol), and the mixture stirred at room temperature for 2 h. Solvent was removed under vacuum with the exclusion of moisture. The white solid residue was dissolved in dichloromethane (20 mL) and treated with diphenylmethanol (392 mg, 2.13 mmol) and triethylamine (226 mg, 2.24 mmol). The solution was stirred at room temperature overnight, washed with 5% HCl (20 mL), and then brine (20 mL). The organic phase was dried (magnesium sulfate) and solvent removed under vacuum to afford a white solid which was recrystallized from ethyl acetate/hexane yielding 519 mg (62%) of **10**, mp 145−146 °C. $^1$H NMR: $\delta$ 7.10−7.40 (m, 20, ArH), 6.48 (d, 1, CH, $J$ = 9.3 Hz), 3.00 (d, 4, $CH_2$, $J$ = 16.2 Hz); IR: 1208, 987, 695 $cm^{-1}$. Anal. ($C_{27}H_{25}NO_2P$) C, H, N.

**Diphenylmethyl Dibenzylphosphinothioate, 13.** To a suspension of **12** (200 mg, 0.81 mmol) in dichloromethane (5 mL) under an atmosphere of nitrogen were added triethylamine (86 mg, 0.85 mmol) and α-phenyl benzenemethanethiol (166 mg, 0.83 mmol) followed by diethylcyanophosphonate (136 mg, 0.81 mmol). The mixture was stirred at room temperature for 24 h, diluted with dichloromethane (20 mL), and washed with water (2 × 20 mL). The organic phase was dried (sodium sulfate) and solvent removed under vacuum to afford crude **13** which was purified via column chromatography (ethyl acetate/hexane, 1:4) to afford **13** (195 mg, 56%) as fine needles, mp 154−156 °C (ethyl acetate/hexane). $^1$H NMR: $\delta$ 7.10−7.40 (m, 20, ArH), 5.77 (d, 1, CH, $J$ = 9.8 Hz), 3.05 (d, 4, $CH_2$, $J$ = 14.6 Hz). IR: 1495, 1204, 1195, 697 $cm^{-1}$. Anal. ($C_{27}H_{25}OPS$) C, H.

**Determination of Inhibition Constants.** The hamster dihydroorotase domain was overexpressed in *E. coli* strain SØ1263/pCW25 and purified as previously described.[6] The pure recombinant DHOase was stored in 20 mM K.Hepes (pH 7.3), 0.1 mM $ZnCl_2$, 1 mM DTT, and 30% (v/v) glycerol at −80 °C and had a specific activity for the reverse reaction (L-dihydroorotate → *N*-carbamyl-L-aspartate) of 2.22 μmol *N*-carbamyl-L-aspartate formed/min/mg protein.

The inhibition constants of **4** and **5** were determined using 10 concentrations of inhibitor ranging from 0 to 60 μM. The reaction mixture contained 50 mM K.Hepes (pH 7.4), 5% (v/v) glycerol, L-[2-$^{14}$C]DHO (20 μM, 54 Ci/mol), and inhibitor (0−60 μM) in a total volume of 25 μL.[6] The reaction mixture was preincubated at 37 °C for 5 min, the reaction was then initiated by addition of DHOase (2 μL, 1.0 ng protein), and the enzymic activity was assayed (DHO → CA-asp) as previously described.[6,9] The pure DHOase was diluted in enzyme buffer [20 mM K.Hepes (pH 7.3), 10% (v/v) glycerol, and 1 mM DTT] to achieve less than 10% conversion of DHO to CA-asp in 15 min. Duplicate determinations were made for each data point. Initial reaction velocities were determined by linear regression from three samples taken at 5 min intervals. $K_i$ values with associated standard errors were determined by nonlinear regression to the velocity equation describing competitive inhibition (eq 1) using the program DNRP53,[19] where $v$ is the measured initial rate, $V_{max}$ is the maximal rate (2.22 μmol/min/mg protein), $S$ is the DHO concentration (20 μM), $I$ is the inhibitor concentration, $K_m$ is the Michaelis constant for DHO (5.02 μM), and $K_i$ is the inhibition constant.

$$v = \frac{VS}{K_m(1 + I/K_i) + S} \quad (1)$$

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