The above nitroacid (6 g, 19 mmol) and NaOH (1.5 g, 37 mmol) were dissolved in water (20 mL), and the solution was kept at 90 °C for 5 h. The cooled mixture was neutralized with HCl, and the resulting bright yellow precipitate of 5-bromo-6-methyl-3-nitroanthranilic acid (**69**; 5.1 g, 97% yield) was collected and washed with water. A sample was crystallized from EtOAc/petroleum ether as orange needles: mp 205–206 °C; ^1H NMR (CD_3SOCD_3) δ 8.29 (s, 1 H, H-4), 7.33 (br s, 2 H, NH_2), 2.45 (s, 3 H, Me). Anal. ($C_8H_7BrN_2O_4$) C, H, N. The crude acid was dissolved in 2.5 equiv of dilute aqueous NaOH and hydrogenated over Pd/C to effect debromination and nitro-group reduction. The resulting crude 2,3-diamino-6-methylbenzoic acid was condensed with benzaldehyde by the method detailed above to give 2-phenyl-5-methyl-1*H*-benzimidazole-4-carboxylic acid (**57**).

A similar sequence from 5-bromo-6-methylisatin[24] gave 2-phenyl-7-methyl-1*H*-benzimidazole-4-carboxylic acid (**59**): mp 304–306 °C; ^1H NMR (CD_3SOCD_3) δ (12.10 (s, 1 H, COOH), 8.50–6.90 (m, 6 H, phenyl protons and NH), 7.75 (d, *J* = 7.5 Hz, 1 H, H-5), 7.25 (d, *J* = 7.5 Hz, 1 H, H-6), 2.65 (s, 3 H, Me). 2-Phenyl-6-methyl-1*H*-benzimidazole-4-carboxylic acid (**58**) was prepared similarly from 5-methyl-3-nitroanthranilic acid:[25] mp 298–300 °C; ^1H NMR (CD_3SOCD_3) δ 8.90–7.00 (m, 8 H, aromatic protons and NH), 2.52 (s, 3 H, Me).

Preparation of 1-Methyl-2-phenyl-1*H*-benzimidazole-4-carboxylic Acid (40). A solution of 3-chloro-2-nitrobenzoic acid (10 g, 49 mmol) in 10% aqueous methylamine (100 mL) was heated at 100 °C for 4 days in a bomb. The resulting solution was evaporated to dryness under reduced pressure to remove excess methylamine, and the residue was redissolved in water. The solution pH was adjusted to 2–3 with concentrated HCl, and extraction with EtOAc gave the crude product (10 g) as a red solid containing several products by TLC. Repeated crystallization from diethyl ether gave 3-(methylamino)-2-nitrobenzoic acid (2.5 g, 26% yield). A sample was recrystallized from diisopropyl ether as red needles, mp 199.5–180 °C. Anal. ($C_8H_8N_2O_4$) C, H, N. Reduction and coupling with benzaldehyde as above gave 1-methyl-2-phenyl-1*H*-benzimidazole-4-carboxylic acid (**40**), mp 154–154.5 °C. Anal. in Table III. Reaction with *N,N*-dimethylethylenediamine as described above gave the free base of **7** as an oil: ^1H NMR ($CDCl_3$) δ 10.20 (t, *J* = 4.9 Hz, 1 H, CONH), 8.16 (dd, *J* = 1.2, 7.5 Hz, 1 H, H-5), 7.83 (m, 2 H, H-2′), 7.55 (m, 3 H, H-3,4′), 7.45 (dd, *J* = 1.2, 8.0 Hz, 1 H, H-7), 7.35 (dd, *J* = 7.5, 8.0 Hz, 1 H, H-6), 3.885 (s, 3 H, NMe), 3.695 and 3.68 (2 t, *J* = 6.5 Hz, 2 H, $CONHCH_2$), 2.64 (t, *J* = 6.5 Hz, 2 H, CH_2NMe_2), 2.33 (s, 6 H, NMe_2).

Similar treatment of 2-chloro-3-nitrobenzoic acid gave the known[26] 2-(methylamino)-3-nitrobenzoic acid, which was used to prepare 3-methyl-2-phenyl-1*H*-benzimidazole-4-carboxylic acid (**41**). Coupling with *N,N*-dimethylethylenediamine as above gave the free base of **6** as an oil: ^1H NMR ($CDCl_3$) δ 8.215 (dd, *J* = 1.0, 8.0 Hz, 1 H, H-5), 8.09 (m, 2 H, H-2′), 7.89 (m, 3 H, H-3′,4′), 7.685 (dd, J = 1.0, 7.4 Hz, 1 H, H-7), 7.575 (dd, *J* = 7.4, 8.0 Hz, 1 H, H-6), 7.49 (t, *J* = 5.0 Hz, CONH), 3.95 and 3.965 (2 t, *J* = 6.6 Hz, 2 H, $CONHCH_2$), 2.92 (t, *J* = 6.1 Hz, CH_2NMe_2), 2.64 (s, 6 H, NMe_2).

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Analogues of Carbamyl Aspartate as Inhibitors of Dihydroorotase: Preparation of Boronic Acid Transition-State Analogues and a Zinc Chelator Carbamylhomocysteine

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Dihydroorotase (DHO) catalyzes the conversion of carbamyl aspartate (CA) to dihydroorotate (DO) in the de novo pyrimidine biosynthetic pathway. Few effective inhibitors of DHO have been reported, and thus blockade of this reaction has not been widely pursued as a strategy for development of antitumor agents. Utilizing two mechanism-based strategies, we have designed and prepared potential DHO inhibitor analogues of CA. One strategy replaced the γ-carboxyl moiety of CA with a boronic acid. This substitution yields compounds which form stable charged tetrahedral intermediates and mimic the enzyme–substrate transition state. Preparation of the boronic acid analogues of CA and its carboxylic acid esters focused on a Curtius rearrangement as a key step following a malonic ester synthesis. This was followed by carbamoylation of the free amine under nonaqueous neutral conditions with $Si(NCO)_4$. The ethyl ester was a competitive inhibitor of DHO with an apparent K_i of 5.07 μM, while the nonesterified analogue and the methyl ester were not effective inhibitors. None of the compounds were cytotoxic against L1210 cells in culture. An active-site-directed sulfhydryl-containing zinc chelator was also prepared. This analogue irreversibly inhibited the enzyme, but it also was ineffective in L1210 growth inhibition.

Inhibitors of pyrimidine and purine biosynthetic enzymes are useful antineoplastic agents.[1] Of the pyrimidine biosynthetic enzymes, dihydroorotase (DHO, EC 3.5.2.3, L-5,6-dihydroorotate amidohydrolase) has been least explored as a target for antimetabolite antitumor agents because of the paucity of effective DHO inhibitors. DHO catalyzes conversion of *N*-carbamylaspartic acid (CA) to dihydroorotate (DO, Figure 1) in the third step of de novo pyrimidine biosynthesis. In mammalian cells DHO activity resides on the multifunctional protein abbreviated CAD. The reversible cyclization of CA to DO catalyzed by DHO proceeds through a reactive tetrahedral transition state.[2] Kelly et al.[3] reported that mammalian DHO contains one

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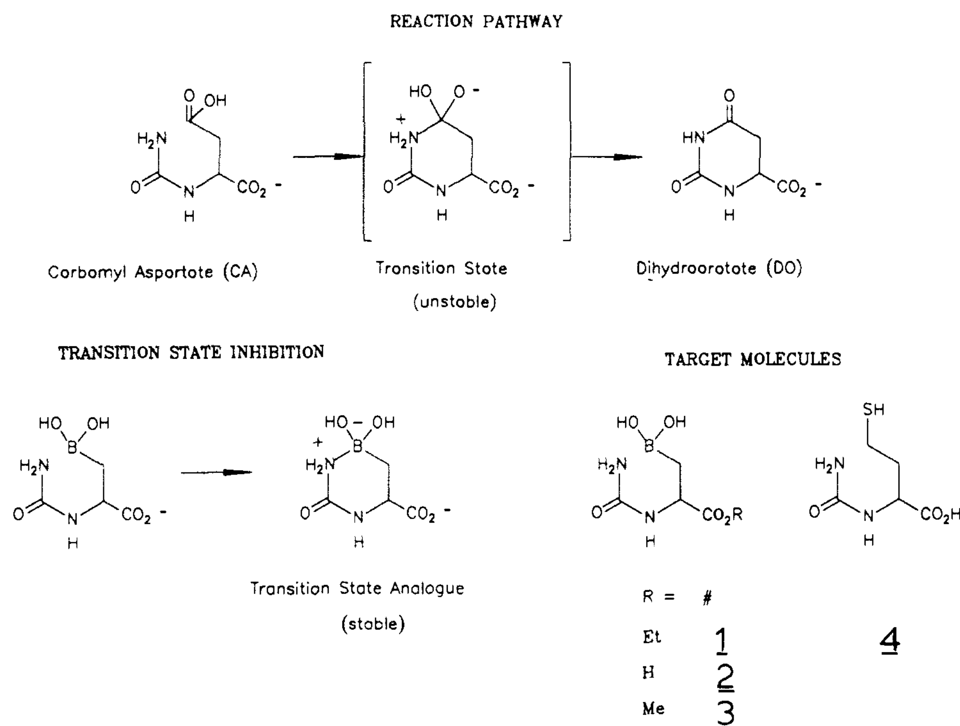

Figure 1. Presumed reaction mechanism for the conversion of CA to DO, compared with the transition-state analogue inhibition scheme for boronic acids. Target molecules are also shown. Sulfhydryl compound 4 is designed to chelate the zinc ion in the active site of DHO.

tightly bound and catalytically essential Zn atom. We have selected two mechanism-based approaches to the design of DHO inhibitors, those being preparation of boronic acid containing transition-state analogues and active-site-directed chelator substrate analogues.

Substitution of boronic acid for carboxylic acid moieties in substrate peptides has previously yielded potent serine protease inhibitors.[4,5] The rationale for such compounds was the well-characterized formation of tetrahedral transition states analogous to that formed with DHO in the enzymatic reaction. Unlike the unstable sp^3 carbon species, the analogous tetrahedral boronic acid structures are stable (Figure 1) and thus inhibit the target enzyme.[6] On the basis of this concept, and successful preparation of protease inhibitors,[4] we have prepared CA analogues **1**–**3**, which possess a boronic acid in place of the γ-carboxyl moiety (Figure 1). On the basis of the presence of zinc in the enzyme, we also prepared the chelator substrate analogue **4** (Figure 1). Herein we report the synthesis, preliminary DHO inhibition studies, and murine leukemia line L1210 growth inhibition studies for these compounds.

Results and Discussion

Synthesis of Compounds. Preparation of CA analogue **1** required introduction of an amino group α to the carboxyl group and β to the boronic acid of precursor molecules. To accomplish this, a Curtius rearrangement was employed as a key reaction in a malonic ester synthesis we previously described.[7] In that reaction scheme, an intermediate isocyanate was trapped with benzyl alcohol to produce the Cbz-protected intermediate **5** (Scheme I). Hydrogenolysis of **5** with H_2, 5% Pd/C produced **6** as a well-behaved solid. Carbamoylation of **6** with $Si(NCO)_4$ produced **7** in 82% yield.[8] The pinacol ester was removed by a transesterification with diethanolamine in acetone or acetone/ethyl acetate to yield **8**, purified by methanol/ethyl acetate recrystallization. The reaction did not proceed if solvents contained significant amounts of water. Passing an aqueous solution of **8** over cation-exchange resin (CO_2H form) produced ethyl ester **1** as a white solid after lyophilization. However, if sulfonic acid resin was used in place of the carboxylic acid resin, **1** was retained on the column, whereas acid **2** was not. Acid **2** was prepared by hydrolysis of the ethyl ester of **8** in aqueous base. The ester was especially labile due to the presence of β-boronic acid (behaving as a Lewis acid), and hydrolysis was complete within 15 min at 0 °C. There was concern that acid **2** would form five-member ring structures such as **9** at physiological pH (e.g., 7.4). NMR data was assessed to determine if cyclization was occurring upon ionization of **2**. The NMR spectrum of acid **2** (D_2O, pH ~3) revealed a doublet triplet pattern, indicating free rotation about the $NCH-CH_2$ carbon–carbon bonds. Potassium salt **9**, prepared by passing the hydrolysis solution through a carboxylic acid resin (H^+ form), had a double doublet pattern for the CHN proton. The BCH_2 protons of **9** were nonequivalent and were shifted upfield from the CH_2 resonances of **2** to 0.9 and 0.5 ppm. Each proton was a double doublet pattern from both vicinal and geminal coupling, suggesting cyclization of the carboxylate oxygen with the boron. Titration of **2** with KOH gave pK_a values of 2.5 and 8.5 for the carboxyl moiety and for the first boronic acid OH group, respectively. At pH 6–8, the NMR spectrum of **2** revealed only the more complex pattern derived from K^+ salt **9**. In solutions of pH <3, the simpler doublet–triplet pattern was observed. Neither ethyl ester **1** nor methyl ester **3** gave NMR spectra consistent with the

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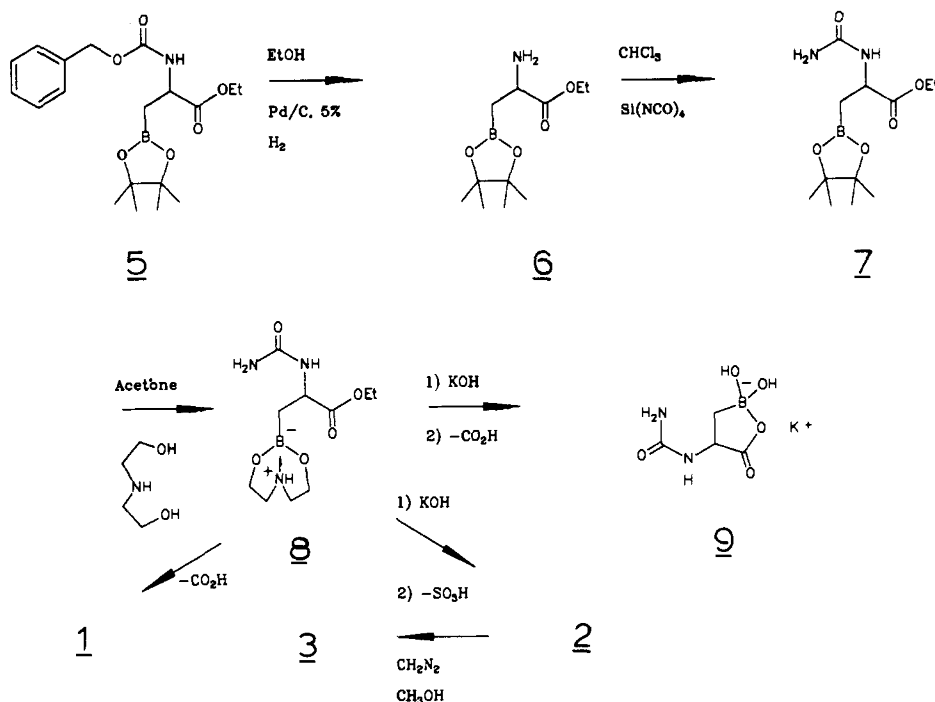
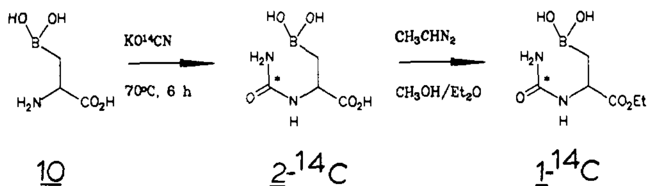
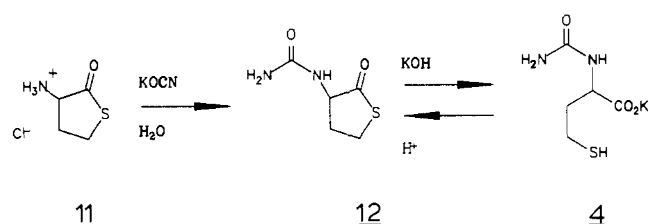
Scheme I



Scheme II



Scheme III



five-member ring structure. These data indicate that cyclization was occurring with the carboxy anion.

Methyl ester **3** was prepared by treatment of a methanolic solution of **2** with an ethereal solution of diazomethane. However, attempts to recrystallize the diethanolamine adduct of **3** as for **8** resulted in hydrolysis of the methyl ester. In aqueous buffered solutions, hydrolysis of the methyl ester was complete within 2 h at pH 9. At pH 6.5, only 10–20% hydrolysis was observed after 2 h. Attempts to recrystallize **3** from methanol, ethanol, and ethyl acetate–ethanol were not successful. NMR spectra and elemental analysis indicated the material was sufficiently pure for preliminary evaluation.

Ethyl ester **1** could be prepared by an alternate route as shown in Scheme II. This route was used to prepare radiolabeled material for stability and pharmacologic studies. Reactions were first optimized with unlabeled materials. Initial attempts to incorporate ^{14}C into the urea carbon of **2** with $Si(NCO)_4$ (from KOCN) were unsuccessful, most likely because it was not possible to purify intermediate products and reagent $Si(NCO)_4$ on a microscale. Ethyl ester **1** was prepared from acid **2**, which was in turn prepared directly from 2.5 equiv of **10**, obtained as previously described, by reaction with aqueous KOCN in 75% yield.[9] Excess **10** was required to consume the KOCN. Molar equiv ratios of **10** and KOCN produced product in only 34% yield. Ethyl ester **1** was obtained after treating **2** with ethereal diazoethane in methanol solutions. Carrier ethyl ester **1** was added and the ethyl ester was purified as the diethanolamine adduct. Ethyl ester **1** was isolated in pure form after passing a solution of the diethanolamine adduct through ion-exchange resin (CO_2H form) and lyophilizing the fractions containing product.

Carbamylhomocysteine analogue **4** was prepared as shown in Scheme III by treating homocysteine thiolactone hydrochloride (**11**) with 1 equiv of KOCN. Carbamyl thiolactone **12** crystallized from the solution and was recovered in 85% yield as an analytically pure solid. Treatment of **12** with KOH produced salt **4**. The free acid could be prepared by acidification of **4** with ion-exchange resin (SO_3H form), but was readily converted back to thiolactone **12** upon drying (vacuum dessicator, 5 h) and was thus not used.

DHO Enzyme Inhibition. Inhibition of DHO was evaluated in the biosynthetic direction using the CAD protein. Free acid analogue **2** did not inhibit DHO at concentrations ≤6 mM. The NMR and titration data discussed above showed that the solution structure of **2** was a 5-membered ring formed with the carboxyl anion and the boron. That structure would not be expected to bind to DHO.

While methyl ester **3** inhibited DHO, repeated experiments did not provide reproducible apparent K_i data. Degradation of **3** due to ester hydrolysis in the enzyme-inhibition cocktail clearly accounted for some of the difficulty in obtaining useful data. In marked contrast, ethyl ester **1** analogue was a competitive inhibitor of the DHO

(9) This preparation was modified from a method for preparation of radiolabeled CA for DHO assays (ref 2). In the original procedures, buffered solutions were used. No desired product **2** was isolated from reactions which utilized buffered conditions.

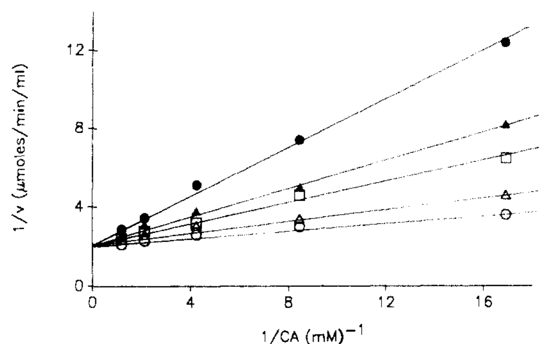


Figure 2. Double-reciprocal plot of dihydroorotase activity at variable concentrations of L-carbamyl aspartate (59, 118, 235, 469, and 833 μM) and ethyl ester 1 concentrations: (○) no analogue, (△) 30 μM analogue, (□) 60 μM analogue, (▲) 106 μM analogue, and (●) 239 μM analogue.

Table I. Murine L1210 Growth Inhibition

compound	concn, μM	cell survival 0 h	24 h	8 h
borocarbamylethyl aspartate	250	114	66	80
PALA	100	96	28	22

reaction as demonstrated by double-reciprocal plots (Figure 2). The apparent K_i (5.07 ± 0.4 μM, pH 6.0) was 10-fold lower than the K_m obtained for CA (K_m = 50 ± 3 μM, pH 6.0). The ethyl ester was stable in the enzyme-inhibition cocktail. While this manuscript was in preparation, Adams et al.[10] reported a competitive inhibitor of DHO [*cis*-4-carboxy-6-(mercaptomethyl)-3,4,5,6-tetrahydropyrimidine-2(1*H*)-one] with an apparent K_i of 140 nm at pH 7.4 and 8.5. These researchers assayed enzyme inhibition in the reverse direction (ring opening). Christopherson and Jones[2] reported that the K_i for orotate was approximately 20-fold higher at the same assay pH when assayed in the forward direction as compared to the reverse direction. In light of those observations, we believe there may be little difference in the potency of 1 and the Adams et al.[10] inhibitor.

Carbamylhomocysteine (4) inhibited DHO in a time-dependent manner with an approximate pseudo-first-order rate for inactivation of 460 × 10^3 min^{-1} at 3 μM as compared to 350 × 10^3 min^{-1} at 50 μM cysteine. It is interesting to note that the Adams et al.[10] sulfhydryl-containing compound was a competitive inhibitor of DHO, but the homocysteine analogue 4 inactivated the enzyme.

Murine Leukemia L1210 Cytotoxicity. Compounds 1–4 and 6 were evaluated for cell growth inhibitory activity against L1210 leukemia cells in depleted media. PALA [*N*-(phosphonoacetyl)-L-aspartic acid], an aspartate transcarbamylase inhibitor cytotoxic against L1210 cells in culture,[11] was included in all experiments as a positive control. Only ethyl ester 1 had minimal activity (Table I). The lack of cytotoxicity when ethyl ester 1 was incubated with L1210 cells was disappointing in light of the DHO inhibition observed with this molecule. Additional studies pointed toward two possible explanations for the minimal cytotoxicity. The uptake of radiolabeled ethyl ester 1 was evaluated under growth inhibition conditions. Uptake of labeled 1 was (0.09%) at a concentration of 210 μM after 24-h incubation with L1210 cells. Radiolabeled thymidine uptake was 18% under identical conditions. Further, while ethyl ester 1 was stable in buffer at 37 °C, the half-life of 1 in the depleted incubation media was 1.5 h at 37 °C. Thus, limited cellular uptake and degradation in incubation media both may have reduced the cytotoxicity of 1.

Conclusion

Antimetabolite inhibitors of de novo pyrimidine biosynthesis have been evaluated as antitumor agents for every enzyme in the pathway except DHO. A number of these molecules, such as the aspartate transcarbamylase inhibitor PALA, have possessed sufficient preclinical antitumor activity to warrant clinical trials.[12] In addition, these molecules are of potential interest as biochemical modulators based on the knowledge of pyrimidine biosynthetic pathways. DHO inhibitors have not been evaluated either as cytotoxics or biochemical modulators because no molecules described to date have been effective inhibitors of this enzyme. Adams et al.[10] recently published data with a DHO inhibitor which lacked in vitro antitumor activity, presumably due to metabolism or chemical degradation.

We designed and prepared several boronic acid analogues of CA, one of which was a competitive inhibitor of DHO. Lack of DHO inhibition by the target boronic acid analogue 2 was explained by formation of a 5-membered ring which would not be expected to interact with the active site of DHO. Inconsistent results were obtained with the methyl ester of 2 possibly due to hydrolysis. Successful DHO inhibition was observed with ethyl ester 1, consistent with formation of a stable tetrahedral inhibitor–enzyme adduct. Similar efforts to inhibit DHO based on substitution of sulfur and phosphorus for carbon (rather than boron) were not effective,[13] presumably because the bulk of these atoms limit access of the compounds to the active site.

The lack of cytotoxicity observed following incubation of 1 with L1210 cells was most likely due to degradation in the incubation medium and/or limited uptake. We are currently preparing analogues which may overcome these limitations.

Experimental Section

Sources of reagents include Aldrich Chemical, Sigma, and CMS, Inc. Reagents were used as received unless otherwise noted. $Si(NCO)_4$ was prepared according to a literature procedure.[8] $KO^{14}CN$ was obtained from ARC, Inc. (St. Louis, MO) with a specific activity of 50 mCi/mmol. NMR spectrum were obtained on an IBM NR80 spectrometer at 80.13 MHz for ^1H and 20.15 MHz for ^{13}C. Proton spectra are reported as ppm downfield from tetramethylsilane (TMS) (δ scale) with either TMS or acetone (2.0 ppm) as internal standard. *J* values are in hertz. Mass spectra were obtained on a Kratos MS50 in either electron impact or fast atom bombardment (FAB) mode, or on a Bio-ion plasma desorption instrument. Elemental analyses were obtained from Galbraith Labs (Knoxville, TN).

Compounds were tested in a cell growth assay against murine L1210 leukemia as has been described.[11] Cells (1 × 10^5) were grown 24 h before drug (in buffer) was added. An aliquot of cells was counted at 0, 24, and 48 h in a hemacytometer. Data are expressed as percent cell survival relative to control.

Preparation of the Cbz amino boronic ester 8 and spectral characteristics of the amino acid 9 have been previously described.[7]

Ethyl 2-Amino-3-(2,3,4,5-tetrahydro-3,3,4,4-tetramethyl-2,5-dioxaborolyl)propionate (6). Z-amino boronic ester 5 (1 g, 2.7 mmol) was dissolved in ethanol and the flask was flushed with N_2. Pd/C (5%, 150 mg) was added to the flask and H_2 was

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bubbled through the mixture. The reaction was followed by TLC, observing the disappearance of 8 (R_f 0.25, 30% EtOAc/hexane). Upon completion (usually 1–2 h) the flask was flushed with N_2 and the catalyst was removed by filtration, through either a celite pad or a Whatman #52 filter paper. After evaporation of the solvent, the white solid was isolated in 80–90% yield and had identical spectral characteristics as previously described for **6** prepared in low yield by an alternate method.[4] This material was used without further purification.

Ethyl 2-(Carbamylamino)-3-(2,3,4,5-tetrahydro-3,3,4,4-tetramethyl-2,5-dioxaborolyl)propionate (7). Freshly prepared **7** (1 g, 4.1 mmol) was dissolved in ethanol-free $CHCl_3$ and treated with 0.22 g (1.1 mmol) of $Si(NCO)_4$.[8] The solution was brought to reflux for 15–30 min. After cooling, the $CHCl_3$ was evaporated and 0.5 mL of H_2O was added, warmed, then 3 mL of isopropyl alcohol was added. After cooling, the solid was removed by filtration through a course, scintered-glass filter and the solid was rinsed with isopropyl alcohol. After evaporation of the solvents and drying over $CaSO_4$ under vacuum, 1.02 g of a white solid (89% yield) was obtained. The product resisted recrystallization attempts and was converted to the diethanolamine adduct **8** for completion of characterization. mp: 216–217 °C. NMR ($CDCl_3$): δ 4.1 (q, J = 9, 2 H, CH_2CH_3), 3.8 (b t, J = 9, 1 H, NCHCO), 1.2 (s, 12 H, pinacol), 1.0 (d, J = 9, 2 H, CH_2B). MS: m/e M^+ + 1 287 (100), 244 (50), 187 (85).

Ethyl 3-(5-Aza-2,8-dioxa-1-boracyclooctanyl)-2-(carbamylamino)propionate (8). Pinacol ester **7** (1.1 g, 4 mmol) was dissolved in acetone (or EtOAc) and treated with 0.4 g (4 mmol) of diethanolamine. A white solid formed rapidly. The solution was placed in a refrigerator overnight. The white solid was collected and recrystallized from methanol to yield 0.63 g (50%) of hydrated material. mp: 230 °C dec. NMR ($D_2O/(CD_3)_2SO$): δ 1.0 (t, J = 10, 3 H, CH_3), 0.8 (m, 2 H, CH_2B), 2.8 (t, J = 9, 4 H, NCH_2), 3.5 (t, J = 10, 4 H, CH_2O), 3.9 (g, J = 10, 2 H, CH_2CH_3), 4.0 (m, 1 H, CHN). Anal.: C, H, N.

Ethyl ester **2** was obtained from diethanolamine adduct **8**. Adduct **8** (100 mg, 0.38 mmol) was dissolved in H_2O and passed through a Dowex Ag 200 × 8 column (CO_2H form, 2 mL of swollen resin), and the resulting solution was lyophilized to give 60 mg (79%) of product as a partial hydrate. mp: 250 °C dec. ^1H NMR (D_2O, acetone): δ 1.0 (d, J = 9, 2 H, CH_2B), 1.05 (t, J = 9, 3 H, CH_3), 3.95 (q, J = 9, 2 H, CH_2CH_3), 4.05 (t, J = 9, 1 H, CHN). Plasma desorbtion MS: M^+ 204. FAB MS: m/e 204 (55), 186 (40), 132 (100). Anal.: C, H, N.

3-Borono-2-(carbamylamino)propionic Acid (2). Diethanolamine adduct **8** (100 mg, 0.38 mmol) was treated with 1.2 mL (3 equiv) of 1 M KOH for 15 min at 0 °C. The solution was passed through a Bio-Rex ion-exchange 50 × 8 column (SO_3H form, 1 mL of swollen resin). The water was lyophilized and 55 mg of solid was obtained (82% yield) after tritriation with acetone. mp: 200 °C wax, 230 °C dec. ^1H NMR (D_2O, acetone): δ 1.0 (d, J = 10, 2 H, BCH_2), 1.1 (t, J = 10, 1 H, CHN). ^{13}C NMR (D_2O, DMSO): δ 25 (b, BC), 55 ($NCCO_2$), 162 (NCN), 184 (CO_2). FAB MS: m/e M^+ 176, 158 ($-H_2O$). Anal.: C, H, N.

Methyl 3-Borono-2-(carbamylamino)propionate (3). Acid **2** was treated with diazomethane in CH_3OH. The solvent was evaporated and the ester was tritiated with acetone to give a white solid in 75% yield. mp: 159–162 °C. ^1H NMR (D_2O, acetone): δ 1.0 (d, J = 9, 2 H, CH_2B), 3.6 (s, 3 H, CH_3), 4.1 (t, J = 9, 1 H, CHN), 4.7 (b s, 5 H, HOD). FAB MS: m/e M^+ − HNCO 149 (15), 83 (100). Anal.: C, H, N.

***N*-Carbamylhomocysteine Thiolactone (12).** Homocysteine thiolactone hydrochloride (**11**) (7.7 g, 50 mmol) was mixed with KOCN (4.1 g, 1 equiv) in 5 mL of H_2O at room temperature. Product slowly precipitated from solution and was collected by filtration after standing overnight to give 4.3 g (56% yield) of product. mp: 150–152 °C. ^1H NMR [$(CD_3)_2SO$]: δ 2 (m, 1 H), 2.3 (m, 1 H), 3.3 (m, 2 H), 4.3 (m, 1 H), 5.7 (b s, 214), 6.3 (b d, 14). ^{13}C NMR [$(CD_3)_2SO$]: δ 207.1 (C(O)S), 159.11 (urea), 60.1 (HNC), 31.8 (CH_2S), 27.4 (CH_2CH_2S). Anal.: C, H, N, S.

Potassium *N*-Carbamylhomocysteine (4). Thiolactone **12** was treated with 1.1 equiv of 1 M KOH for 1 h at room temperature. The solution was passed through a Dowex 200 × 8 column (CO_2H form) and the product was isolated by lyophilization of the solution as a hygroscopic white solid. mp: >250 °C. NMR [$(CD_3)_2SO$]: δ 1.9 (m, 2 H), 2.2 (m, 2 H), 4.1 (m, 1 H), 5.5 (s, 2 H), 6.2 (b d, 1 H). Anal.: C, H, N, S.

Ethyl 3-Borono-2-([^{14}C]carbamylamino)propionate (1-^{14}C). Boro aspartate **10** (6.6 mg, 50 μmol) was added to 1.62 mg of $KO^{14}CN$ [2 μmol, 50 mCi/mmol, or 25 mCi/mmol as carrier added (0.81 mg KOCN + 0.81 mg $KO^{14}CN$)] in a total volume of 0.7 mL of doubly distilled H_2O. The solution was heated with stirring at 65–70 °C for 3–4 h. Upon cooling, the solution was passed through a Dowex 200 × resin. The product **2**-^{14}C was collected, lyophilized, and used without further purification. The ester **1**-^{14}C was prepared by treating a methanolic solution of **2**-^{14}C with ethereal diazoethane at 0 °C and evaporating the solvents. Ester **1** (4 mg) was added as carrier, and the solid was heated with 1 equiv of diethanolamine in CH_3OH. A crystal of **8** was added to initiate crystallization, and the sample was placed in the freezer overnight. The mother liquor was removed via a capillary pipette, and the solid was rinsed with a drop of cold methanol. The solid was taken up in H_2O and passed through a Bio-Rex AB 90 × resin (H^+) and the product was isolated after lyophilization of the H_2O. Spectral data indicate **1** prepared in this manner is identical with **1** prepared as outlined in Scheme I.

L1210 Cytotoxicity. L1210 cells were maintained in standard culture medium: Dulbecco's modified Eagles medium (DMEM) containing fetal-calf serum (10%) and L-glutamine (0.29 mg/mL). Culture medium was replenished and passaged three times weekly. Cells in log-phase growth were harvested for growth inhibition assays.

For growth inhibition assays, cells (~1 × 10^5 cells/mL) were suspended in depleted media (24 h) and to each flask was added vehicle (DMSO), PALA, or DHO inhibitors. Viable cells (trypan blue) were counted at indicated times and expressed as percent of control cell counts. Three flasks were used for each drug concentration and six control flasks were used for each experiment.

DHO Enzyme Assay. CAD protein was isolated from a mutant Simian virus 40 transformed Syrian hamster cell line (165-23) which overproduces this protein[14] (this cell line was generously provided by David R. Evans, Wayne State University, Detroit, MI). *N*-[^{14}C]Carbamylaspartic acid (10–15 mCi/mmol) was prepared according to the method of Christopherson and Jones.[2]

DHO was assayed in the biosynthetic direction (36 mM MES–NaOH, pH 6) utilizing thin-layer chromatography on polyethyleneimine cellulose plates, with 0.19 M LiCl as eluent to separate CA and DO.[2] Radioactivity was quantitated by means of a Radiometric TLC scanner. Apparent K_i values were determined from double-reciprocal plots according to literature methods.[15] Boro aspartic acid analogue **2** was inactive against DHO. Ethyl ester analogue **1** inhibited DHO with an apparent K_i value of 5.07 ± 0.4 μM. Carbamylhomocysteine analogue **4** inhibited DHO in a time-dependent manner with an approximate pseudo-first-order rate constant for inactivation of 460 × 10^3 min^{-1} at 3.0 μM.

L1210 Cell Uptake. Murine L1210 cells in depleted media were exposed to 210 μM [^{14}C]ethyl ester **1** (6.9 μCi/μmol) or to 455 nM [^{14}C]thymidine (7.9 μCi/μmol) for 24 h. Radioactivity incorporated into cells was evaluated by the method of Wohlhueter et al.[16]

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