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# STABLE MULTISUBSTRATE ADDUCTS AS ENZYME INHIBITORS

# POTENT INHIBITION OF ORNITHINE DECARBOXYLASE BY *N*-(5′-PHOSPHOPYRIDOXYL)-ORNITHINE

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## Summary

The synthesis of several *N*-(5′-phosphopyridoxyl)-amino acids is described. These compounds, analogs of the Schiff base intermediate involved in enzyme-catalyzed decarboxylation, are potent inhibitors of the cognate amino acid decarboxylases. Kinetic studies using partially purified rat liver ornithine decarboxylase, have shown that *N*-(5′-phosphopyridoxyl)-ornithine inhibits the enzyme in a non-competitive manner with respect to both ornithine and pyridoxal-5′-phosphate. These findings suggest that the inhibitor binds to the holoenzyme active site in place of the Schiff base intermediate.

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One of the major advances in the design of new drugs in the past few decades has been the use of so-called metabolite analogs, or antimetabolites, as reversible or irreversible inhibitors of selected enzymes [1,2]. However, a deficiency of this approach is that the antimetabolite usually blocks most enzymes with which the natural metabolite is associated in the cell. This lack of in vitro specificity by simple co-enzyme analogs is amply demonstrated by: (1) inhibition of pyridoxal phosphate-dependent decarboxylases by 4-bromo-3-hydroxybenzyloxyamine (Brocresine, NSD-1055) [3], (2) inhibition of several enzymes involved in de novo synthesis of purines by 6-mercaptopurine [4] or (3) the inhibition of several methylases by the 7-deaza analog of *S*-adenosyl-homocys-

Abbreviations: PLP, pyridoxal 5′-phosphate; *S*-Ado-met, *S*-adenosyl-L-methionine; Cbz-, benzyloxycarbonyl-; Boc-, *t*-butoxycarbonyl-.

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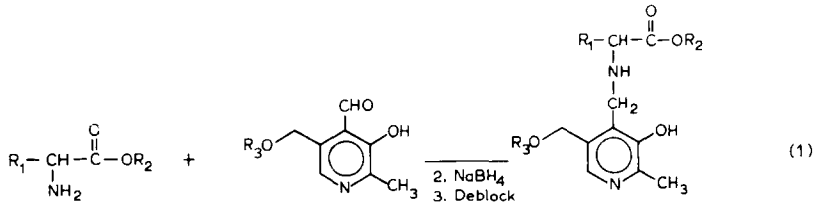
teine [5]. Some selective toxicity is achieved in vivo by fortuitous differences in metabolism of the drug, cellular localization of enzymes, and related phenomena [1]. However, the differences which can be exploited are many and varied, and generally not easily predicted for any individual drug. One way to achieve the desired specificity in vitro is to exploit differences in the transition states of the various reactions involving the same metabolite. Although this approach has successfully been used to develop very potent inhibitors of single substrate enzymes [6,7], only a limited effort has been made to use this approach to develop inhibitors for enzymes involving more than one substrate [8,9]. While much has been written about the magnitude of inhibition constants observed with transition state analogs [6,7,10], little attention has been focused on the increased specificity to be expected with such compounds. Therefore, we have begun to study the effects of this type of analog on closely related enzymes which utilize a common co-enzyme or metabolite as one of the substrates. This paper describes the synthesis and kinetic properties of a new inhibitor of the enzyme which catalyzes the decarboxylation of ornithine (ornithine decarboxylase, EC 4.1.1.17), the rate-limiting step in polyamine biosynthesis [11].

The enzymes which are involved in polyamine biosynthesis are well studied, both in bacterial [12], and mammalian cells [13]. The role of pyridoxal-5′-phosphate (PLP) in the decarboxylation of ornithine is well established [12,13], and a similar role for PLP has been proposed in the decarboxylation of *S*-adenosylmethionine (*S*-Ado-Met) in rat prostate [14,15], although this is still a controversial question [16–18]. It is known that the pyridoxal analog, 4-bromo-3-hydroxybenzyloxyamine, is an inhibitor of both of these enzymes [15,19]. Therefore, in order to obtain a specific inhibitor for each of these two decarboxylases involved in the biosynthesis of polyamines, we chose to synthesize the reduced Schiff base adducts analogous to the Schiff bases involved in the enzyme-catalyzed decarboxylation. Recent studies have shown that methylglyoxal bis (guanylhydrazone) (MGBG) is a potent and apparently




(1)

1

2

3

a, $R_1$ = $-(CH_2)_3$ NHCbz, $R_2$ = H
b, $R_1$ = $-(CH_2)_3$ NHCbz, $R_2$ = $-CH_2C_6H_5$
c, $R_1$ = $-(CH_2)_3$ NHBoc, $R_2$ = H
d, $R_1$ = $-(CH_2)_3$ NHBoc, $R_2$ = $CH_3$
e, $R_1$ = $-(CH_2)_2$ S(+)(CH3)-Ad, $R_2$ = H
f, $R_1$ = $-(CH_2)_4$ NHBoc, $R_2$ = H

a, $R_3$ = H
b, $R_3$ = $-P(=O)-(OH)_2$

a, $R_1$ = $-(CH_2)_3NH_2$, $R_2$ = H, $R_3$ = $-P(=O)(OH)_2$
b, $R_1$ = $-(CH_2)_3NH_2$, $R_2$ = $R_3$ = H
c, $R_1$ = $(CH_2)_4NH_2$, $R_2$ = H, $R_3$ = $-P(=O)(OH)_2$

specific inhibitor of *S*-adenosylmethionine decarboxylase [20]. Several publications have described the synthesis of *N*-(pyridoxyl) amino acids [21,22] and *N*-(5′-phosphopyridoxyl) amino acids [22—25], but only three of these papers [23—25] have included any data on the inhibition of enzymes by these compounds. We have used the method of Severin et al. [23] to prepare the reduced Schiff base adducts 3 by use of appropriately blocked amino acids (adduct 1), and either pyridoxal (adduct 2) or pyridoxal-5′-phosphate (adduct 2b) as shown in Eqn. 1.

## Material and Methods

*Enzymes*

Pyridoxal (adduct 2a) and pyridoxal-5′-phosphate (PLP, adduct 2b) were from Sigma. *N*-δ-Cbz-L-ornithine (Cbz: benzyloxycarbonyl-) was from Schwarz/Mann, *N*-δ-Boc-L-ornithine (Boc-: *t*-butoxycarbonyl-) and *N*-ε-Boc-L-lysine were from Bachem Inc. DL-[1-$^{14}$C]Ornithine (7.66 Ci/mol), L-[U-$^{14}$C]lysine (0.06 Ci/mol), were from New England Nuclear, and *S*-adenosyl-L-[1-$^{14}$C]methionine (1.32 Ci/mol) was from Amersham-Searle. The DL-[1-$^{14}$C]ornithine was dissolved in 2.5 ml of 0.7% perchloric acid, heated to 57°C for 30 min, neutralized with 1 M KOH and diluted to 5 ml with 100 mM Tris buffer, pH 7.6 at 37°C containing 0.5 mM EDTA. This treatment reduced blank values to 100—200 cpm. Enzymes were isolated and routinely assayed according to literature procedures: lysine decarboxylase from *Escherichia coli* [26], *S*-adenosylmethionine decarboxylase from rat prostate [15] and tyrosine aminotransferase from rat liver [27]. Ornithine decarboxylase was purified 50-fold according to the following modification of the procedure of Ono et al. [28], and was induced by injecting 80 rats (150—180 g) with dexamethasone phosphate [29] (kindly supplied by Merck, Sharp and Dohme; Rahway, New Jersey) 1 mg/100 g body wt. The animals were sacrificed 3 h later, the livers removed and homogenized in 2 vol. of 0.25 M sucrose, 10 mM mercaptoethanol, 10 mM Tris buffer (pH 7.0) at 4°C. All subsequent operations were carried out at 4°C. The homogenate was centrifuged at 23 000 × *g* for 20 min and the supernatant was then centrifuged at 100 000 × *g* for 1 h. The pH of the supernatant was adjusted to pH 4.6 by the slow addition of 2 M acetic acid. The solution was centrifuged at 20 000 × *g* for 10 min and the precipitate homogenized in 0.1—0.3 times the original volume with 50 mM Tris buffer (pH 7.6) containing 0.075 mM PLP, 10 mM mercaptoethanol, 0.5 mM EDTA. The pH of the homogenate (generally 5.5—6.0) was adjusted to 7.0 with 50 mM Tris base and dialyzed against 300 vol. of 10 mM Tris buffer described above, with one change of buffer over a period of 8 h. The dialyzed material was centrifuged at 20 000 × *g* for 10 min and the supernatant stored at −20°C. This supernatant, which contained 4.5 g protein, was then applied to a DEAE-cellulose column (2.6 × 40 cm) equilibrated with the dialysis buffer. The column was washed with approx. 150 ml of dialysis buffer containing 0.05 M KCl and then with 150 ml of dialysis buffer containing 0.1 M KCl. The enzyme was eluted with a linear KCl gradient generated by placing 0.1 M KCl (750 ml of dialysis buffer) in the mixing chamber and 0.4 M KCl (750 ml of dialysis buffer) in the reservoir at a flow rate of 60 to 70 ml/h. The active fractions were pooled, concen-

trated by ultracentrifugation and stored at −70°C. The overall recovery of activity was approx. 65% and specific activity of the enzyme was 58 nmol $CO_2$ formed per h per mg protein.

For routine assay of ornithine decarboxylase activity the method of Friedman et al. [30] was used, except that the concentration of PLP was maintained at 75 μM. For kinetic studies with saturating levels of PLP or ornithine, the following modification of the standard assay [30] was used so that higher specific activities could be obtained at reasonable cost. The incubation mixture contained in a final volume of 0.155 ml: 5 μmol Tris buffer (pH 7.6 at 37°C), 0.1 μmol EDTA, 1.0 μmol mercapthoethanol, 0.1 μmol [1-$^{14}$C] ornithine (0.76 μCi), enzyme solution, and appropriate amounts of adduct 3a and PLP (or ornithine) as indicated. The incubations were carried out in glass vials (12 × 35 mm) placed in glass scintillation vials containing 1.5 ml of 0.02 M citric acid for efficient heat transfer. The scintillation vials were closed with plastic caps supporting a plastic well containing 0.2 ml of a 2 : 1 mixture of ethylene glycol monoethyl ether and monoethanolamine. After 1 h at 37°C, the reactions were stopped by injecting 0.2 ml of 2 M citric acid into the incubation mixture. The samples were incubated overnight at 25°C with shaking and the radioactivity determined by placing the wells into 10 ml of scintillation cocktail (33% ethanol and 4.2% liquifluor in toluene) and assaying in a Packard Tri-carb liquid scintillation spectrometer. All values were corrected for the appropriate blanks (generally 100–200 cpm). The recovery of $^{14}CO_2$ as determined from $NaH^{14}CO_3$ was 89% for this method. Protein was measured by the method of Ross and Schatz [31], and velocities are in units of nmol $CO_2$ formed per h per mg protein.

*Chemistry*

Our initial synthetic studies employed *N*-δ-Cbz-L-ornithine (adduct 1a) for coupling with adduct 2. Unfortunately the free acid was only partially soluble in a solution of methanolic KOH [23], and thin-layer chromatography analysis of the reaction mixture revealed that approx. 50% of the starting material, adduct 1a, remained. In order to circumvent the solubility problems, the benzyl ester adduct 1b [32] was coupled with adduct 2b in methanolic KOH [23], and the fully blocked reduced Schiff base purified to homogeneity by chromatography on silica gel. However deblocking of this pure product in various acidic and/or basic media resulted in considerable cleavage of the 5′-phosphate or lactam formation, rather than the desired adduct 3a. Next we turned our attention to a more labile N-blocking group, and chose the t-butoxy carbonyl (Boc) group. *N*-δ-Boc-L-ornithine methyl ester (addult 1d) [33] was coupled with pyridoxal phosphate in the usual manner [23], and hydrolysis of the Boc-group with $CF_3COOH$, followed by saponification of the methyl ester again led to lactam formation, as observed with the adduct derived from adduct 1b. While attempting to study the time course of the reduction reaction in aqueous media, it was observed that adduct 1c was considerably more soluble in $H_2O$ than was adduct 1a. Therefore it appeared that the free acid adduct 1c might be used in the coupling-reduction reaction, and this proved to be the method of choice for the synthesis of adduct 3. Thus, coupling of either adduct 1c or adduct 1f with adduct 2, followed by reduction with $NaBH_4$ gave a crude

reduced Schiff base, which was sufficiently pure for further transformations. Deblocking the $\omega$-amino group with $CF_3COOH$, followed by chromatography on Dowex 50 ($H^+$ form) gave the desired adduct 3 with only trace amounts of impurities. An analytical sample could be obtained by preparative thin-layer chromatography on cellulose in butanol/acetic acid/water (12 : 3 :5).

A typical preparation of adduct 3 is as follows: Pyridoxal-5′-phosphate hydrate (265 mg, 1 mmol) was dissolved in a solution of KOH (112 mg, 2 mmol) in 5 ml anhydrous methanol. To the resulting yellow solution was added a solution of the appropriate $\omega$-Boc amino acid (1 mmol) in 8 ml methanol containing 56 mg (1 mmol) KOH. The clear yellow reaction mixture was cooled in an ice bath to 0°C and after 20 min total reaction time, was treated with sodium borohydride (approx. 40 mg) until the bright yellow solution turned almost colorless and did not fade upon further additions of sodium borohydride. The reaction mixture was allowed to stir in the ice bath for an additional 15 min, and then was neutralized at 0°C with acetic acid to pH 6. The solvent was removed in vacuo at room temperature resulting in a yellow, bubbly, glass-like solid. This residue was treated in an ice bath with 10 ml of chilled trifluoroacetic acid to remove the Boc- protecting group. After 15 min the trifluoroacetic acid was removed in vacuo at room temperature. Residual traces of trifluoroacetic acid were removed via a toluene azeotrope, resulting in an off-white gummy residue. The gummy residue was dissolved in 10 ml of water and the foggy aqueous solution was extracted once with ether. The resulting clear, faintly yellow, aqueous solution was applied directly on a Bio Rad AG 50W-X4 (100—200 mesh, $H^+$ form) column (1.6 × 24 cm). The column was washed with approx. 300 ml of water and the product eluted with 1 M $NH_4OH$ in 5-ml fractions. Typically, the product came off in fractions 33—40. The combined peak fractions were lyophilized to give a pale yellow solid in good yield. The physical properties of adduct 3 are given in Table I.

All attempts to prepare adduct 3 from *S*-Ado-met and adduct 2 failed due to the facile intramolecular cyclization of *S*-Ado-met and/or its reduced Schiff base adduct, adduct 3, in basic media to give 5′-methylthioadenosine in addition to homoserine lactone and/or its reduced Schiff base adduct. This can be readily demonstrated by thin-layer chromatography of the reaction mixture and comparison with authentic 5′-methylthio-5′-deoxyadenosine. This reaction is well documented in aqueous media [34,35], and the decreased stability of sulfonium ions in non-aqueous media [36] presumably results in a very fast intramolecular cyclization under the reaction conditions to give the observed products rather than the desired adduct of *S*-Ado-met and adduct 2.

## Results and Discussion

The reduced Schiff base adducts 3, were tested as inhibitors of the decarboxylases which utilize PLP, and either ornithine or lysine, in addition to several other PLP-dependent decarboxylases and aminotransferases. Pyridoxamine and pyridoxamine-5′-phosphate were also tested as inhibitors of ornithine decarboxylase. These data, summarized in Table II, clearly demonstrate the requirement for the phosphate and amino acid portions of adduct 3 in order to obtain potent inhibition of the enzymes studied. In addition, it has been found

TABLE I

PHYSICAL PROPERTIES OF ADDUCT 3

| Compound [a] | Thin-layer chromatography $R_F$ [b] | 0.1 M $H^+$ $\lambda_{max}$ (nm) | 0.1 M $OH^-$ $\lambda_{max}$ (nm) | NMR, $\delta$ [c] | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | $C_1H$ | $C_2CH_3$ | $C_5'H$ | $C_4'H$ | $\alpha CH$ | $\beta CH_2$ | $\gamma CH_2$ | $\delta CH_2$ | $\epsilon CH_2$ |
| 3a adduct | 0.21 | 295 | 307,244 | 7.57 (s) | 2.31 (s) | 4.70, 4.80 | 4.23 (s) | 3.60 (t) | 1.90 (m) | 1.90 (m) | 2.90 (t) | — |
| 3b adduct | 0.40 | 293 | 308,243 | 7.59 (s) | 2.33 (s) | 4.55 (s) | 4.13 (s) | 3.38 (t) | 1.82 (m) | 1.82 (m) | 3.02 (t) | — |
| 3c adduct | 0.20 | 295 | 308,244 | 7.64 (s) | 2.39 (s) | 4.83 [d] | 4.31 (s) | 3.68 (t) | 1.73 (bd.m) | 1.73 (bd.m) | 1.73 (bd.m) | 2.99 (t) |

[a] All compounds were very hygroscopic. Elemental analyses of purified material (see text) indicated that the samples contained water and/or acetic acid.

[b] Cellulose chromatograms (Eastman No. 6065) in *n*-butanol/acetic acid/water (12:3:5). All compounds showed one spot at the indicated $R_F$ which was both ultraviolet positive and ninhydrin positive. In addition, the spot for adducts 3a and 3c reacted with the Hanes-Isherwood reagent for phosphate esters [47].

[c] NMR spectra were obtained on a Varian T60A spectrometer. The sample was in $^2H_2O$ containing acetone as an internal standard, and chemical shift data are given in ppm downfield from tetramethylsilane.

[d] Presumed downfield peak of $C_5'H$ doublet is obscured by the $H^2HO$ peak.

TABLE II

EFFECT OF ADDUCT 3 ON VARIOUS PLP-DEPENDENT ENZYMES

Enzymes used are abbreviated as follows: ODC, L-Ornithine decarboxylase (EC 4.1.1.17); LDC, L-lysine decarboxylase (EC 4.1.1.18); SAMDC, *S*-adenosyl-L-methionine decarboxylase (EC 4.1.1.50); TAT, Tyrosine aminotransferase (EC 2.6.1.5). Enzymes were isolated and assayed as described in the text.

| Enzyme | [PLP] (mM) | [Amino acid] (mM) [a] | Inhibition [b] (%) | | |
|---|---|---|---|---|---|
| | | | 3a | 3b | 3c |
| ODC [c] | 0.075 | 0.64 (0.07) | 90 | 0 | 85 |
| LDC | 0.05 | 3.0 (1.5) | 75 | 0 | 90 |
| SAMDC | 0 | 0.1 (0.05) | 0 | 0 | n.d. |
| TAT | 0.05 | 6.3 (1.4) | 22 | 8 | n.d. |

[a] $K_m$ (mM) for amino acid given in parentheses.

[b] All inhibitors present at a final concentration of 1.0 mM; n.d., not determined.

[c] 32% inhibition of ornithine decarboxylase was observed in the presence of 1.0 mM pyridoxamine-5'-phosphate as inhibitor in place of 3. No inhibition of ornithine decarboxylase was observed in the presence of 1.0 mM pyridoxamine.

that the *N*-(5'-phosphopyridoxyl)alanine (adduct 3, $R_1 = CH_3$, $R_2 = H$, $R_3 = OP(OH)_2$) is ineffective as an inhibitor of *E. coli* ornithine decarboxylase (T. Karpetsky and P. Talalay, personal communication), thus demonstrating a requirement for the appropriate amino acid side chain ($R_1$) in the adduct 3. The fact that adducts 3a and 3c are both effective against ornithine and lysine decarboxylase is evidence for some flexibility in the binding of these adducts to the enzymes. However, it should be noted that the ornithine adduct, 3a, was consistently more potent than the lysine adduct, 3c, as an inhibitor of ornithine decarboxylase, and conversely, adduct 3c was consistently more potent that adduct 3a as an inhibitor of lysine decarboxylase. In addition, lysine decarboxylase is a bacterial enzyme and its presence has not been demonstrated in any mammalian cells to date. Thus, for in vivo studies in mammalian cells, this lack of absolute specificity is of little consequence. These data are qualitatively similar to those presented in prior publications dealing with inhibition of other PLP-dependent enzymes by *N*-(5'-phosphopyridoxyl)amino acids. Thus, apo-glutamate-aspartate transaminase (EC 2.6.1.1) is similarly inhibited by both *N*-(5'-phosphopyridoxyl)glutamate, and *N*-(5'-phosphopyridoxyl)aspartate [23]. Other PLP-amino adducts are much less effective at concentrations comparable with that of PLP (40 $\mu$M) employed in the standard assay [23]. In the same manner, it has been shown that apo-dopa decarboxylase (aromatic acid decarboxylase; EC 4.1.1.28) is inhibited by both *N*-(5'-phosphopyridoxyl)tyrosine and *N*-5'-(phosphopyridoxyl)phenylalanine, while other PLP-amine adducts are much less effective [25]. The slight inhibition of tyrosine aminotransferase by 1 mM adduct 3a is perhaps due to the phosphate moiety of adduct 3a, since it is known that phosphate ion is an effective inhibitor of this enzyme [27].

More detailed kinetic studies were carried out with adduct 3a and ornithine decarboxylase. Figs 1 and 2 show the inhibition data at saturating levels of ornithine and PLP respectively. These plots lead to values of $K_m$ for ornithine of 70.2 $\mu$M and for PLP of 0.26 $\mu$M, which are in good agreement with literature values of 80 $\mu$M and 0.3 $\mu$M respectively [37]. Replots of these data

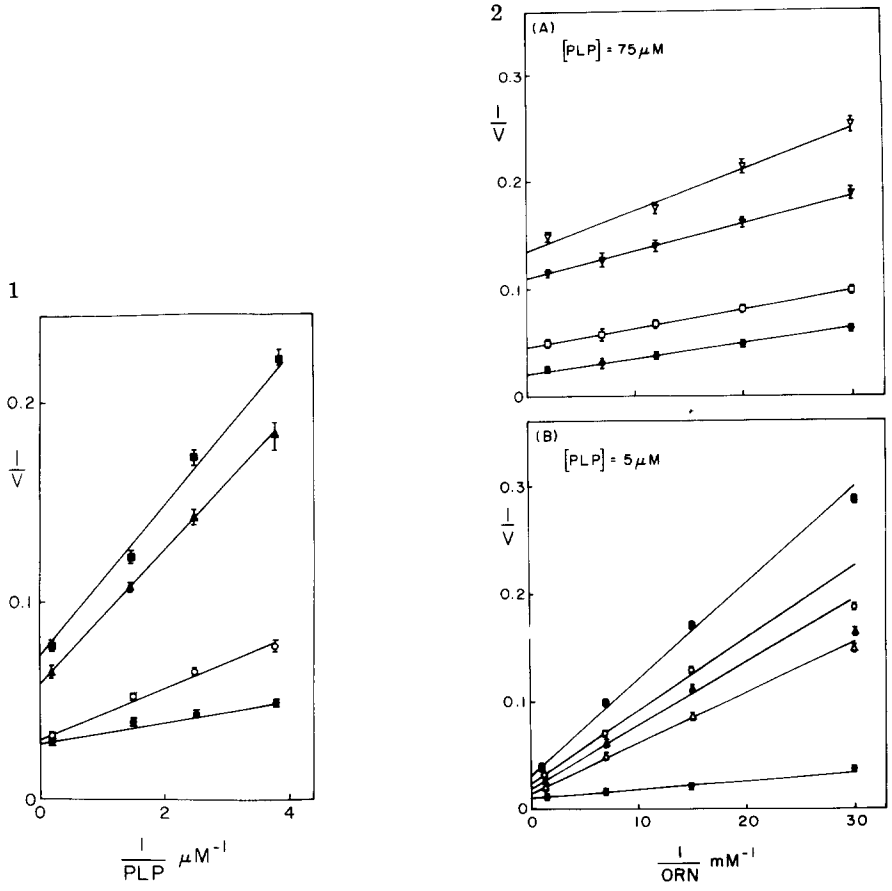
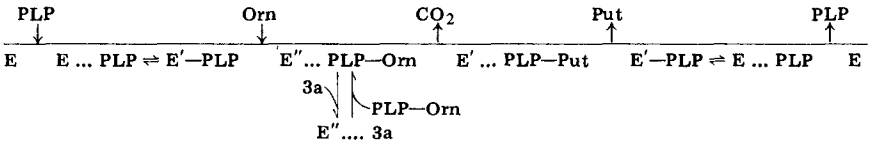



Fig. 1. Lineweaver-Burk plot of kinetic data for the inhibition of ornithine decarboxylase by 0 μM (●), 0.1 μM (○), 0.64 μM (▲), and 1.0 μM (■) adduct 3a, in the presence of 0.6 mM L-ornithine. Ornithine decarboxylase was isolated and purified as described in the text. Enzyme (100 μl) was diluted to 1.2 ml with buffer, and dialyzed against 1 l buffer (10 mM Tris, pH 7.6, 0.5 mM EDTA, 10 mM 2-mercaptoethanol and 50 mM KCl) for 8 h to obtain apo-ornithine decarboxylase.

Fig. 2. Lineweaver-Burk plot of kinetic data for the inhibition of ornithine decarboxylase. Panel A: Inhibitor concentrations were 0 μM (●), 10 μM (□), 50 μM (▼), and 100 μM (▽) adduct 3a in the presence of 75 μM PLP. Panel B: Inhibitor concentrations were 0 μM (●), 0.39 μM (△), 0.64 μM (▲), 1.0 μM (○), and 2.0 μM (■) adduct 3a in the presence of 5.0 μM PLP. Ornithine decarboxylase was isolated and purified as described in the text and then diluted 1 : 200 with buffer prior to assay.

show that the kinetic equations of ornithine decarboxylase inhibition by adduct 3a are very complex. Against varying concentrations of PLP and saturating levels (610 μM) of ornithine (Fig. 1), S-hyperbolic I-linear non-competitive inhibition [38] is observed, whereas against varying concentrations of ornithine and saturating levels (75 μM and 5 μM) of PLP, S-linear I-hyperbolic (Fig. 2A), or S-hyperbolic I-linear (Fig. 2B) non-competitive inhibition is observed. In general, PLP-dependent decarboxylases are thought to bind substrates and products via the ordered sequence shown in Scheme I [39]. Thus, in the present case, PLP would be expected to bind first to the apoenzyme, E, and then form holoenzyme complex (E′ · PLP) between PLP and a presumed lysyl side chain of the protein via an intermediate non-covalently bound enzyme · PLP

SCHEME I




Orn, ornithine; Put, putrescine

complex (E. · PLP). The E′ · PLP complex could then undergo a transimination reaction to form the key intermediate imine between PLP and ornithine (E″ · PLP-ornithine). Decarboxylation, followed by release of products, would then proceed via the steps shown in Scheme I. It is important to incorporate the features of Scheme I into any discussion of the inhibition data. Since adduct 3a is simply the reduced counterpart of PLP-ornithine, it seems reasonable to assume that adduct 3a will bind to the enzyme in the site normally occupied by PLP-ornithine during the decarboxylation. Consideration of Scheme I leads to the conclusion therefore, that adduct 3a must bind to enzyme E″ in order to form the inhibitor E″ · adduct 3a complex analogous to E″ · PLP-ornithine. When appreciable amounts of E′ · PLP are present, as is the case with saturating levels of PLP and limiting amounts of ornithine, it is more difficult for adduct 3a to bind E″ and form the inhibitory complex. This is in contrast to the situation with saturating levels of ornithine and variable, non-saturating concentrations of PLP, where considerable apoenzyme, E, is available and little E′ · PLP is present. Any E′ · PLP which does form is rapidly converted to E″ · PLP-ornithine, in which case adduct 3a can combine with E″ to form the inhibitory complex.

If adduct 3a were acting simply as an analog of either PLP or ornithine in a normal sequential ordered reaction [38], one would predict that the inhibition of ornithine decarboxylase by adduct 3a could be overcome by saturating concentrations of either PLP or ornithine; i.e., a PLP analog would exhibit competitive kinetics against varying concentrations of PLP, etc. It is clear from the data in Figs 1 and 2 that this is not the case in the present work. The non-competitive inhibition observed may be explained by the series of equilibria in Scheme I, in which adduct 3a binds to E″, and not to free apoenzyme E. In the case of several PLP-dependent enzymes [40,41], there are data that support a slow interconversion between conformationally distinct apoenzyme and holo-enzyme via a rapidly formed intermediate of unspecified structure. Therefore it is probable that enzyme E″ differs somewhat in conformation from apoenzyme E. These two enzyme forms would be distinguishable in a steady-state kinetic analysis and therefore non-competitive inhibition by adduct 3a should be observed. This is the case in the inhibition of ornithine decarboxylase by adduct 3a in the presence of both non-saturating (Fig. 1) and saturating (Fig. 2) levels of PLP.

$K_i$ values obtained from these data are 1.2 and 66 $\mu$M in the presence of saturating PLP concentrations of 5 $\mu$M and 75 $\mu$M respectively, and 0.6 $\mu$M in the presence of saturating ornithine (610 $\mu$M) and varying PLP. The large difference in the $K_i$ values obtained in the presence of saturating PLP (Fig. 2)

presumably is a consequence of the requirement that adduct 3a bind to E″, which is less available in the presence of a saturating concentration of PLP and limiting ornithine. However, the formation of E′ · PLP is a reversible process, and at concentrations of adduct 3a approaching the concentration of PLP inhibition is observed as adduct 3a begins to compete with PLP for E″. This leads to $K_i$ values of 1.2 $\mu$M and 66 $\mu$M, which are very similar to concentrations of PLP present in the respective assays (Fig. 2). It should be noted that the apparent competition of adduct 3a and PLP will not lead to competitive inhibition kinetics when varying PLP in the presence of saturating ornithine (Fig. 1). This is because adduct 3a binds to E″, whereas PLP initially added to the reaction mixture binds to apoenzyme E. The lower $K_i$ value obtained against varying amounts of PLP is more indicative of the potential efficacy of the drug in vivo, since the concentration of PLP in a liver homogenate is approx. 28 nmol per g [42] and thus limiting in comparison to the value of approx. 210 nmol per g [43] for the concentration of ornithine. In this regard, it is of interest to note that several analogs of ornithine have been synthesized and tested as ornithine decarboxylase inhibitors [37,44—46]. These compounds are either inactive as ornithine decarboxylase inhibitors [45], or inhibit the enzyme by reacting with PLP, thereby depleting the supply of PLP available for reaction with ornithine prior to decarboxylation. The latter type of inhibition can be reversed by high concentrations of PLP [37,44] although a recent paper has claimed that the inhibition of rat prostate ornithine decarboxylase by $\alpha$-methyl ornithine is not reversed by PLP [46]. However, the authors gave few details concerning their enzyme isolation procedure, and did not report a complete kinetic analysis over a wide range of PLP concentrations [46]. Our results show that inhibition of apo-ornithine decarboxylase by adduct 3a is not reversed by high concentrations of PLP (Fig. 1). In addition, even at saturating levels of PLP where holoenzyme certainly is the predominant species, adduct 3a is still an effective inhibitor of ornithine decarboxylase, although higher concentrations of the drug are required for the reasons discussed above.

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## References

1 Albert, A. (1973) Selective Toxicity, 5th ed., Chap. 8. Chapman and Hall, London
2 Baker, B.R. (1967) Design of Active-Site-Directed Irreversible Enzyme Inhibitors, Wiley, New York
3 Taylor, Jr., R.J., Leinweber, F.J. and Braun, G.A. (1973) Biochem. Pharmacol. 22, 2299
4 Montgomery, J.A. (1965) Prog. Drug Res. 8, 433
5 Coward, J.K., Bussolotti, D.L. and Chang, C.-D. (1974) J. Med. Chem. 17, 1286
6 Wolfenden, R. (1972) Acc. Chem. Res. 5, 10
7 Lienhard, G. (1973) Science 180, 149
8 Lienhard, G. (1972) Ann. Rep. Med. Chem. 7, 249
9 Byers, L.D. and Wolfenden, R. (1973) Biochemistry 12, 2070

10 Schray, K. and Klinman, J.P. (1974) Biochem. Biophys. Res. Commun. 57, 641
11 Cohen, S.S. (1971) Introduction to the Polyamines, Prentice-Hall, Englewood Cliffs, N.J.
12 Tabor, H. and Tabor, C.W. (1972) Adv. Enzymol. 36, 203
13 Williams-Ashman, H.G., Jänne, J., Coppoc, G.L., Geroch, M.E. and Schenone, A. (1972) Adv. Enz. Reg. 10, 225
14 Feldman, M.J., Levy, C.C. and Russell, D.H. (1972) Biochemistry 11, 671
15 Pegg, A.E. and Williams-Ashman, H.G. (1969) J. Biol. Chem. 244, 682
16 Pegg, A. (1974) Biochem. J. 141, 581
17 Manen, C.-A. and Russell, D. (1974) Biochemistry 13, 4729
18 Sturman, J.A. and Kremzner, L.T. (1974) Biochim. Biophys. Acta 372, 162
19 Schrock, T.R., Oakman, N.J. and Bucher, N.L.R. (1970) Biochim. Biophys. Acta 204, 564
20 Williams-Ashman, H.G. and Schenone, A. (1972) Biochem. Biophys. Res. Commun. 46, 288
21 Heyl, D., Harris, S.A. and Folkers, K. (1948) J. Am. Chem. Soc. 70, 3429
22 Ikawa, M. (1967) Arch. Biochem. Biophys. 118, 497
23 Severin, E.S., Gulyaev, N.N., Khurs, E.N. and Khomutov, R.M. (1969) Biochem. Biophys. Res. Commun. 35, 318
24 Khomutov, R.M., Dixon, H.B.F., Vdovina, L.V., Kirpichnikov, M.P., Morozov., Y.V., Severin, E.S. and Khurs, E.N. (1971) Biochem. J. 124, 99
25 Borri-Voltattorni, C., Minelli, A. and Turano, C. (1971) Boll. Soc. Ital. Biol. Sper. 47, 700
26 Sabo, D.L., Boeker, E.A., Byers, B., Waron, H. and Fischer, E.H. (1974) Biochemistry 13, 662
27 Granner, D.K. and Tomkins, G.M. (1970) Methods in Enzymol. 17A, 633
28 Ono, M., Inoue, H., Suzuki, F. and Takeda, Y. (1972) Biochim. Biophys. Acta 284, 285
29 Beck, W.T., Bellantone, R.A. and Canellakis, E.S. (1972) Biochem. Biophys. Res. Commun. 48, 1649
30 Friedman, S.J., Halpern, K.V. and Canellakis, E.S. (1972) Biochim. Biophys. Acta 261, 181
31 Ross, E. and Schatz, G. (1973) Anal. Biochem. 54, 304
32 Losse, G., Jeschkeit, H. and Zaschke, H. (1964) Justus Liebigs Ann. Chem. 676, 232
33 Schröder, E., Petras, H.-S. and Klieger, E. (1964) Justus Liebigs Ann. Chem. 679, 221
34 Cantoni, G.L. (1953) J. Biol. Chem. 204, 403
35 Schlenk, F.E. and DePalma, R.E. (1957) J. Biol. Chem. 229, 1037
36 Coward, J.K. and Sweet, W.D. (1971) J. Org. Chem. 36, 2337
37 Harik, S.I. and Snyder, S.H. (1973) Biochim. Biophys. Acta 327, 501
38 Cleland, W.W., Boyer, P. (ed.) (1970) The Enzymes, 3rd ed., Vol. II, Academic Press, New York, p. 1
39 Snell, E.E., Boyer, P. (ed.) (1972) The Enzymes, 3rd ed., Vol. VI, Academic Press, New York, p. 217
40 Churchich, J.E. and Farrelly, J.G. (1969) J. Biol. Chem. 244, 3685
41 O'Leary, M.H. and Malik, J.M. (1972) J. Biol. Chem. 244, 7097
42 Sevigny, S.J., White, S.L., Halsey, M.L. and Johnston, F.A. (1966) J. Nutr. 88, 45
43 Bloxan, D.L. (1972) Brit. J. Nutr. 27, 233
44 Rahiala, E.L., Kekomaki, M., Jänne, J., Raina, A. and Raiha, R. (1971) Biochim. Biophys. Acta 227, 337
45 Skinner, W.A. and Johansson, J.G. (1972) J. Med. Chem. 15, 427
46 Abdel-Monem, M.M., Newton, N.E. and Weeks, C.E. (1974) J. Med. Chem. 17, 447
47 Hanes, E.S. and Isherwood, F.A. (1949) Nature 164, 1107