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Inhibition of DOPA decarboxylation by analogues of tryptophan

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Aromatic L-amino acid decarboxylase (EC 4.1.1.28) has been characterized as the enzyme responsible for the conversion of 3,4-dihydroxyphenylalanine (DOPA) to 3,4-dihydroxyphenylethylamine (DA), a reaction first demonstrated by Holtz *et al.* [1] in 1938 with kidney extracts from rabbits and guinea pigs. The enzyme was subsequently shown to be widely distributed in mammalian tissues and to require pyridoxal phosphate [2]. It has been purified from hog kidney by Christenson *et al.* [3] and characterized in terms of optimal cofactor and substrate concentrations and medium pH by these authors and by Werle and Aures [4]. In addition, a variety of aromatic L-amino acids (both natural and unnatural) have been shown to have activity as substrates [3, 5–7].

One of the more interesting aspects of this enzyme has been the controversy surrounding its ability to act as the decarboxylation system for indoleamines such as tryptophan as well as for phenylalanines [8, 10]. There is no doubt that aromatic L-amino acid decarboxylase preparations purified from hog kidney [3] have considerable activity toward a variety of aromatic L-amino acids, including analogues of tryptophan [3, 9, 11–13]. Some analogues of tryptophan that are poor substrates for decarboxylation have been shown, not unexpectedly, to be inhibitors of the decarboxylation of tryptophan, as well as of other amino acids [13, 14].

The finding of Ehringer and Hornykiewicz [15] that patients with Parkinson's disease had subnormal levels of DA in the substantia nigra and the subsequent discovery and development of L-DOPA as a therapeutic agent have stimulated new interest in inhibitors of the decarboxylation of this amino acid [16–18]. A variety of compounds have been found to be inhibitors of the decarboxylation system *in vitro*, including hydrazine derivatives [18–20], α-methyl amino acids [10, 21] and hydroxycinnamic acids [22–24]. A recent report from this laboratory has indicated that the benzo[b]thiophene and 1-methylindole analogues of tryptophan are effective inhibitors of the decarboxylation of tryptophan and phenylalanine *in vitro* [13]. This paper reports on the activity of several analogues of tryptophan as inhibitors of the decarboxylation of L-DOPA.

DL-DOPA-2[^{14}C] (50 mCi/m-mole) and DA-2[^{14}C] (46 mCi/m-mole) were purchased from Amersham/Searle Corp., Chicago, IL, and L-DOPA[^{3}H] (general label, 5.92 Ci/m-mole) and DA[^{3}H] (general label, 9.34 Ci/m-mole) were purchased from New England Nuclear Corp., Boston, MA. DL-DOPA, DL-tryptophan and pyridoxal phosphate were purchased from Sigma Chemical Co., St. Louis, MO; DA was purchased from Nutritional Biochemical Corp., Cleveland, OH; and 5-hydroxytryptophan (5-HTP), α-methyltryptophan, L-DOPA, 3,4-dihydroxyphenylacetic acid, 3,4-dihydroxyphenylethanol, 3-methoxy-4-hydroxyphenylethanol, and homovanillic acid were purchased from Regis Chemical Co., Chicago, IL. The benzo[b]thiophene analogues of tryptophan, β-(3-benzo[b]thienyl)-α-alanine; 5-HTP, β-(5-hydroxy-3-benzo[b]thienyl)-α-alanine; α-methyltryptophan, α-methyl-β-(3-benzo[b]thienyl)-α-alanine; and *l*-methyltryptophan were supplied as racemates by Dr. E. Campaigne, Chemistry Department, Indiana University, Bloomington, IN. Pargyline hydrochloride was kindly supplied by Abbott Laboratories, and α-methyl-DOPA (Aldomet) was supplied by Merck, Sharpe & Dohme.

All chemicals and reagents used were purchased from commercial sources. Ion exchange resin (Amberlite CG-50, type 2, 200–400 mesh) was obtained from Mallinckrodt Chemical Co., St. Louis, MO. Adult, male, Swiss–Webster mice (18–25 g) were purchased from Murphy Breeding Laboratories, Plainfield, IN. Hog kidneys were obtained fresh from the Winterlein Meat Packing Co., Bloomington, IN.

The procedure of Christenson *et al.* [3] was followed through the second ammonium sulfate precipitation with minor modifications. The final precipitate was dissolved in 150 ml of 0.01 M 2-mercaptoethanol, dialyzed overnight against 0.01 M 2-mercaptoethanol, and then frozen until use. Enzyme protein was determined by the method of Lowry *et al.* [25].

The frozen enzyme was allowed to thaw at room temperature prior to use. All incubations were done in triplicate, using an incubation mixture modeled after Creveling and Daly [26] and consisting of: 0.2 ml of 0.5 M NaH_2PO_4 buffer (pH 7,0), 0.1 ml pyridoxal-5-phosphate (final concentration 10^{-4} M), 0.01 ml pargyline HCl (final concentration 1.25×10^{-5} M), 0.02 ml enzyme, 0.01 ml DOPA, and distilled water or inhibitor to a final volume of 1.0 ml. All incubations were done at 37° for 10 min under air in an Aminco constant temperature shaking incubator. The mixture was pre-incubated for 3 min before addition of the substrate. Each incubation vessel contained 0.2 μCi DL-DOPA-2-[^{14}C]. Inhibitors, when used, were added in 0.5 ml volume 1 min prior to the addition of the substrate; 0.5 ml H_2O was deleted from the original reaction mixture to allow the final volume to remain at 1 ml. The enzymatic reaction was stopped by the addition of 0.1 ml of 50% trichloroacetic acid (TCA). After addition of 0.1 ml of 1×10^{-3} M DA as a carrier plus 10 mg ascorbic acid, 2 ml of 0.5 M KH_2PO_4 (pH 6.5) and NH_4OH were then added to obtain a pH of 6.5. The mixture was transferred to centrifuge tubes and spun at 37,000 rev/min in an IEC model HN centrifuge for 15 min. One ml of the incubate was then added to the ion exchange columns. Zero time points were prepared by the addition of 0.1 ml of 50% TCA just prior to addition of the substrate.

The columns were 1-ml disposable pipets cut to 12 cm in length with a 12-ml disposable syringe used as a reservoir. A three-way plastic stopcock connected the reservoir to the pipet. The resin (Amberlite CG-50), 150 mg/column, was prepared by the method of Hirs *et al.* [27] and used in the Na^+-form at pH 6.5.

After the 1-ml sample loading, the columns were washed with 8.0 ml of distilled water, then with 1 ml of 2 N HCl and 0.5 ml of the eluant was pipetted into a polyethylene vial containing 15 ml of scintillation fluid, prepared by mixing 15 g of 2,5-bis-2-(5-tert-butylbenzoxazolyl) thiophene (BBOT), 280 g napthalene, 2000 ml toluene, and 1400 ml of 2-methoxyethanol. Each sample was counted twice for 10 min in a Packard Tricarb liquid scintillation spectrometer, model 2425. Recovery of DA over the range 10^{-7}–10^{-4} M was 82–93 per cent under these conditions, with no interference from DOPA. K_m, V_{max} and K_i were calculated by linear regression analysis of unweighted double reciprocal ($1/V$ vs $1/[S]$) data, using computerized procedures.

For experiments *in vivo*, drugs were made up in 0.5% methyl cellulose suspensions. Pretreated mice received 300 mg/kg of inhibitor, i.p., 1.5 hr prior to an oral dose of 200 mg/kg of L-DOPA (1.97 mCi/m-mole). Control mice

Table 1. Inhibition of DOPA decarboxylation by various compounds *in vitro**

Compound	% Inhibition	K_i (M)
Tryptophan	0.0	
l-Methyltryptophan	10.8	
β-(3-Benzo[b]thienyl)-α-alanine	31.1	1.9×10^{-3}
α-Methyl-DOPA	51.7	7.7×10^{-4}
5-Hydroxytryptophan	83.2	2.1×10^{-4}
β-(5-Hydroxy-3-benzo[b]thienyl)-α-alanine	93.5	5.8×10^{-5}
α-Methyltryptophan		3.7×10^{-4}
α-Methyl-β-(3-benzo[b]thienyl)-α-alanine		7.0×10^{-4}

* Purified hog kidney enzyme was used, as described in the text. Per cent inhibition values were computed from studies with substrate concentration of 5×10^{-5} M and inhibitor concentration of 10^{-3} M. Values for K_i were determined from multiple combinations of substrate (5×10^{-5} to 10^{-3} M) and inhibitor (10^{-4} to 10^{-3} M) concentrations. All values were derived from four to six replicate runs with a fixed 10-min incubation time.

received only an oral dose of 200 mg/kg of L-DOPA (1.97 mCi/m-mole). Groups of six mice were sacrificed by decapitation 1 and 2 hr after DOPA administration. Tissue samples were removed, rinsed with saline and frozen immediately. The samples were assayed by a procedure modified after Taylor and Laverty [28].

The extraction process was tested with authentic DOPA, DA, norepinephrine(NE), 3-methoxy-4-hydroxyphenylethanol (chosen to represent the neutral/alcohol metabolites) and homovanillic acid (chosen to represent an acidic metabolite). Concentrated samples of each fraction from the column procedure were spotted on Whatman No. 1 paper, developed using two solvent systems—butanol–acetic acid–H_2O (25:4:10) and isopropanol–2 N HCl (65:35)—and scanned (Packard model 7200 radiochromatogram scanner). Authentic compounds were detected with iodine vapor and showed separation into three fractions, corresponding to DOPA, DA + NE, and HVA + 3-methoxy-4-hydroxyphenylethanol.

Using the purified hog kidney enzyme, initial studies showed the reaction for the decarboxylation of DOPA to be linear over 2–10 min of incubation, with K_m values from 1.96×10^{-4} to 2.38×10^{-4} over a range of substrate concentrations of 5×10^{-5} to 1×10^{-3} M, and a preliminary series of inhibition studies was performed. The results (Table 1) show that the benzo[b]thiophene analogue of 5-HTP was the most potent inhibitor (93.5 per cent inhibition); 5-HTP itself was slightly less potent (83.2 per cent), and α-methyl-DOPA produced a 51.7 per cent inhibition.

Detailed kinetic studies were performed with 5-HTP, α-methyl-5-hydroxytryptophan, and the benzo[b]thiophene analogues of these two compounds. The results (Table 1) indicate that the benzo[b]thiophene analogue of 5-HTP was the most potent inhibitor of DOPA decarboxylation in the test system ($K_i = 5.8 \times 10^{-5}$ M). In the case of α-methyltryptophan, replacement of the indole nitrogen atom with a sulfur atom led to a slight decrease in potency. The Lineweaver–Burk plots for the inhibition of DOPA decarboxylation by these compounds are shown in Fig. 1.

In an attempt to further test the efficacy of the most potent inhibitor *in vitro*, mice were pretreated with the benzo[b]thiophene analogue of 5-HTP prior to the administration of L-DOPA, and brain and kidney were removed at 1 and 2 hr for the assay of DOPA, DA/NE, and metabolites as described in the text. Brain levels of total radioactivity and of DA + NE were significantly higher

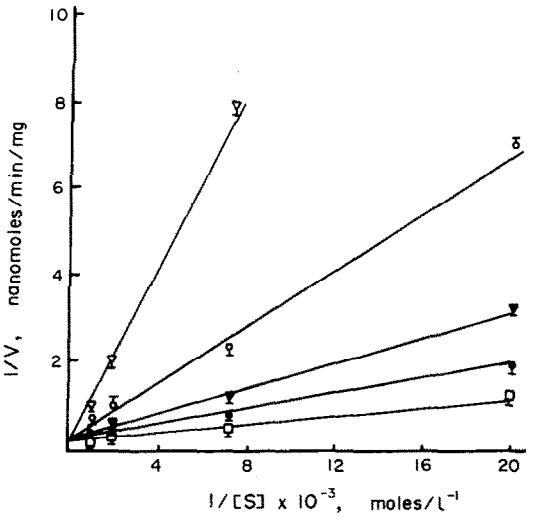

Fig. 1. Lineweaver–Burk plots of inhibition of DOPA (5×10^{-5} M) decarboxylation by various compounds (10^{-3} M). Key: DOPA alone (—□—), α-methyl-β-(3-benzo[b]thienyl)-α-alanine (—●—), α-methyltryptophan (—▼—), 5-hydroxytryptophan (—○—), and β-(5-hydroxy-3-benzo[b]-thienyl)-α-alanine (—▽—). Vertical bars indicate ± S. E. M.

Table 2. Levels of ^{3}H-labeled compounds in mouse brain after [^{3}H]L-DOPA*

Group	Total ^{3}H	L-DOPA	DA + NE	Metabolites
Control (1 hr)	101.6 ± 13.0	42.9 ± 6.9	8.3 ± 1.1	4.1 ± 0.6
Pretreated (1 hr)	146.7 ± 10.0†	57.0 ± 9.4	13.1 ± 2.9†	5.4 ± 0.7
Control (2 hr)	120.4 ± 16.6	51.7 ± 6.4	9.6 ± 0.9	5.8 ± 0.9
Pretreated (2 hr)	109.1 ± 8.3	32.1 ± 4.6‡	7.8 ± 0.9	3.7 ± 0.5‡

* Animals were dosed (with or without pretreatment with the benzo[b]thiophene analogue of 5-HTP), as described in the text. Each value is the mean ± S. E. M. of values obtained from six mice, calculated as nmoles/g.

† Pretreated was significantly greater than control ($P > 0.05$).

‡ Pretreated was significantly less than control ($P < 0.05$).

after 1 hr in mice pretreated with the inhibitor; in contrast, at the 2-hr point, brain levels of L-DOPA and the metabolites were significantly less (Table 2). The only significant difference in kidney values was a lowered level of DA + NE in the pretreated mice killed 2 hr after L-DOPA.

The results presented in this paper indicate that the benzo[b]thiophene analogue of 5-HTP is the most potent inhibitor of DOPA decarboxylation (Table 1), followed in order by α-methyl-DOPA and the benzo[b]thiophene and 1-methylindole analogues of tryptophan; tryptophan itself was inactive. These results are consistent with previous findings that a 5-hydroxy function considerably increases the affinity of tryptophan for the enzyme [5, 16, 29]. One can also compare this to the relative rates of decarboxylation of 5-HTP, tyrosine isomers and tryptophan; 5-HTP and tyrosine isomers are decarboxylated at a rate of 10–40 per cent that of DOPA, while tryptophan is decarboxylated 1–10 per cent that of DOPA [5]. On this basis, the benzo-[b]thiophene analogue of 5-HTP should have the highest affinity, followed by α-methyl-DOPA, tryptophan, and derivatives not having the 5-hydroxy group. The fact that the benzo[b]thiophene analogue of tryptophan is considerably more lipid soluble than tryptophan [30], combined with the fact that it is sterically similar to tryptophan, may facilitate binding to the active site of the enzyme.

Lipid solubility may also explain why α-methyltryptophan has more affinity for the enzyme than tryptophan. 1-Methyltryptophan is practically as lipid soluble as the benzo[b]thiophene analogue, which would also facilitate binding to the enzyme. The fact that the bulk of the methylated indole nitrogen may prevent the compound from being as strongly bound to the enzyme probably dictates the order of affinity indicated in Table 1.

From the kinetic studies presented in Fig. 1, it is clear that substitution of sulfur for the indole nitrogen atom increases the affinity of the enzyme for the 5-hydroxylated compounds, while the same substitution in α-methylated compounds produces the opposite effect. The bulkiness of the α-methyl group may greatly affect the positioning of the structure within the active site, thereby limiting the interaction of the benzo[b]thiophene nucleus with a specific area of the protein. Therefore, when considering α-methylated compounds, steric hinderance may be the limiting factor rather than the lipid solubility of the compounds.

The small duration of effect seen when the benzo[b]-thiophene analogue of 5-HTP was used as a pretreatment *in vivo* prior to administration of L-DOPA is somewhat disconcerting in view of the relative potency of this compound as an inhibitor *in vitro*. However, only a single dose combination was tested, and nothing is known of the rate of degradation and excretion of this inhibitor. Further studies are needed to explore the possible significance of these compounds as inhibitors of DOPA decarboxylation.

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