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Analogues of Fusaric (5-Butylpicolinic) Acid as Potent Inhibitors of Dopamine β-Hydroxylase

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SUMMARY

HIDAKA, HIROYOSHI, ASANO, TOMIKO, AND TAKEMOTO, NOBUTAKA: Analogues of fusaric (5-butylpicolinic) acid as potent inhibitors of dopamine β-hydroxylase. *Mol. Pharmacol.* **9**, 172–177 (1973).

Several derivatives of picolinic acid were effective inhibitors of a purified bovine adrenal dopamine β-hydroxylase [3,4-dihydroxyphenylethylamine, ascorbate:oxygen oxidoreductase (hydroxylating), EC 1.14.2.1]. Inhibition of the enzyme by derivatives of picolinic acid was uncompetitive with the substrate and competitive with ascorbic acid. Among the derivatives of picolinic acid tested, 5-(3′,4′-dihalobutyl)picolinic acid and 5-(3′-halobutyl) picolinic acid were the most potent inhibitors of the hydroxylase, producing 50% inhibition at a concentration of 10 nM. While 5-butylpicolinic acid seemed to have the highest stability constant of the copper chelate among the picolinic acids tested, its inhibitory activity was not the strongest. There was no correlation between the copper-chelating capacity of the picolinic acids and their inhibitory effects on the enzyme. Diethyldithiocarbamate, which is a more effective copper-chelating agent than 5-butylpicolinic acid was a less potent enzyme inhibitor. These data indicate that the inhibition of dopamine β-hydroxylase by picolinic acids was not primarily due to the chelate formation between the compounds and copper of the enzyme. Some of these picolinic acids seemed to be specific inhibitors of the hydroxylase and did not inhibit tyrosinase, a copper-containing enzyme, up to 10,000 times the concentration effectively inhibitory to dopamine β-hydroxylase.

INTRODUCTION

Dopamine β-hydroxylase [3,4-dihydroxyphenylethylamine, ascorbate:oxygen oxidoreductase (hydroxylating), EC 1.14.2.1], the enzyme that catalyzes the conversion of dopamine to norepinephrine (1), is localized in the chromaffin granules in the adrenal medulla (2) and in the catecholamine-containing vesicles in sympathetic nerve terminals (3). Disulfiram, diethyldithiocarbamate, and various aromatic thioureas are inhibitors of this enzyme both *in vivo* and *in vitro* (4–7).

Increasing interest in delineating possible roles of dopamine β-hydroxylase in norepinephrine biosynthesis has prompted a search for compounds which will produce specific inhibition of this enzyme. Hidaka *et al.* (8) reported that 5-butylpicolinic acid (fusaric acid) is an effective hypotensive agent and a potent inhibitor of dopamine β-hydroxylase (approximately 10 times more potent than disulfiram). Terasawa *et al.* (9) used FA[2] clinically and found it to be ef-

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[2] The abbreviations used are: FA, 5-butylpicolinic acid (fusaric acid); Br_2FA, 5-(3′,4′-dibromobutyl)picolinic acid; BrFA, 5-(3′-bromobutyl)picolinic acid; Cl_2FA, 5-(3′,4′-dichlorobutyl)picolinic acid.

fective against human hypertension. These facts have stimulated our interest in the use of analogues of FA as inhibitors of dopamine β-hydroxylase, as hypotensive agents, or as pharmacological tools to alter levels of amines in the nervous system.

MATERIALS AND METHODS

The picolinic acids used in this study were synthesized at the Biological Screening Office, Banyu Pharmaceutical Company, Tokyo. Dopamine β-hydroxylase was isolated from bovine adrenals and highly purified through the ammonium sulfate precipitation, charcoal treatment, calcium phosphate gel treatment, and DEAE-cellulose column chromatography steps as described by Friedman and Kaufman (10). A spectrophotometric assay using tyramine as the substrate was employed to determine enzyme activity (11). The incubation mixture (1 ml) contained the following components: potassium phosphate buffer (pH 5.5), 100 mM; ascorbic acid, 10 mM; fumaric acid, 12 mM; tyramine hydrochloride, 10 mM; enough crystalline catalase to give maximal stimulation of the reaction rate; and 20–40 μg of the purified enzyme. Concentrations of inhibitors were decreased over the range of 100 μM to 1 nM until the inhibition fell below 50%. The inhibitor concentration producing 45–55% inhibition (I_{50}) was determined graphically (12). Mushroom tyrosinase was purchased from Miles Company, Ltd. Its activity was measured by the increase of absorption in the region of 280–300 nm caused by the formation of products. The reaction mixture (3 ml) contained 1 mM L-tyrosine, 100 mM phosphate buffer (pH 6.5), and 0.2 mg of the enzyme. Tyrosinase was incubated for 10 min with or without inhibitor before adding L-tyrosine. The reaction was initiated by adding the substrate, L-tyrosine, at 25°.

Oxine (8-hydroxyquinoline) was employed as a displacing ligand of copper to determine the copper-chelating capacity (the relative stability constant) of the picolinic acids (13). When the concentrations of copper, oxine, and the picolinic acids are 100, 200, and 200 μM, respectively, the relative stability constant (K_d) of picolinic acid and oxine is expressed by Eq. 1:

$$K_d = \frac{(1 - [Cu(Ox)_2])^3}{[Cu(Ox)_2]^3} \qquad (1)$$

where $Cu(Ox)_2$ is the copper-oxine chelate.

The chelate formed between oxine and copper [$Cu(Ox)_2$] has maximal absorption at 390 nm, but copper, oxine, the picolinic acids alone, and the picolinic acid–copper chelates have no absorption at 390 nm. Accordingly, the amounts of the copper-oxine chelate were determined at an optical density of 390 nm when the picolinic acids were added to the solution containing oxine-copper chelate. Reactions were carried out in 95% ethanol–2.5 mM acetate buffer (pH 5.0) at 25°. The copper-chelating capacity (relative stability constant) of diethyldithiocarbamate was determined spectrophotometrically by measuring the decrease of A_{430}, which is the absorption maximum of the diethyldithiocarbamate-copper chelate, using FA as a displacing ligand (13).

RESULTS

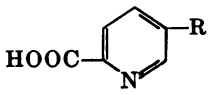
All the picolinic acids tested were found to be inhibitory to some extent. The inhibitory effects of some of the more interesting members of this group upon the enzyme *in vitro* are summarized in Table 1. 5-(3′,4′-Dibromobutyl)picolinic acid, 5-(3′-bromobutyl)picolinic acid, and 5-(3′,4′-dichlorobutyl)picolinic acid were the most active of the inhibitors in this series.

Table 2 summarizes the concentration of $Cu(Ox)_2$ remaining in the absence or the presence of the picolinic acids (200 μM) and the relative stability constants of the picolinic acids and diethyldithiocarbamate. The compound with the higher value in Table 2 forms the more stable copper chelate. Substitution by various radicals at position 5 of picolinic acid did not produce marked changes in the stability constants of their copper chelates, except for O-165 (Table 2). FA has a higher capacity to form a copper chelate (Table 2), but it is not relatively potent as an inhibitor of dopamine β-hydroxylase (Table 1). Although diethyldithiocarbamate was approximately 13 times less active an inhibitor of the enzyme than was FA (Table 1), this substance was a more effective copper-chelating agent than FA

TABLE 1

Inhibition of dopamine β-hydroxylase by picolinic acids

The picolinic acids and diethyldithiocarbamate were added to the incubation mixture, and I_{50} was determined graphically (12) (see MATERIALS AND METHODS).



R	Compound	I_{50}
		nM
$-CH_2-CH_2-CHBr-CH_2Br$	Br_2FA	10
$-CH_2-CH_2-CHBr-CH_3$	BrFA	10
$-CH_2-CH_2-CHCl-CH_2Cl$	Cl_2FA	10
$-CH_2-CH_2-CH_2-CH_3$	FA	76
$-CH_2-S-CS-N(CH_3)_2$	YP-279	77
$-H$	Picolinc acid	800
$-NH-CS-N(CH_3)_2$	B-265	1,000
$-CH_2-O-CO-N(CH_3)_2$	YP-180	5,000
$-SO_2-NH-CH_3$	O-165	80,000
	Sodium diethyldithiocarbamate	1,000

TABLE 2

Spectrophotometric determination of relative stability constants (K_d) of copper chelate of picolinic acids and diethyldithiocarbamate

The picolinic acids (200 μM) were added to a 95% ethanol–acetate buffer (2.5 mM, pH 5.0) containing 100 μM $Cu(Ox)_2$. The molar extinction coefficient of $Cu(Ox)_2$ in this solution was calculated to be 5420. The concentration of $Cu(Ox)_2$ remaining after addition of the picolinic acids was determined by measuring A_{390}. K_d was determined by fitting experimental data to Eq. 1.

Test substance	A_{390}	$Cu(Ox)_2$ remaining	K_d	Log K_d
		μM		
None	0.542	100		
Br_2FA	0.203	37.5	4.63	0.666
FA	0.164	30.3	12.19	1.086
YP-279	0.239	44.1	2.04	0.310
Picolinic acid	0.210	38.7	3.97	0.599
YP-180	0.239	44.1	2.04	0.310
O-165	0.439	81.0	0.013	−1.886
Diethyldithiocarbamate			145,000[a]	5.161

[a] K_d of the copper chelate of diethyldithiocarbamate and oxine was calculated from the K_d of the copper chelate of diethyldithiocarbamate and fusaric acid (see MATERIALS AND METHODS).

(Table 2). As shown in Tables 1 and 2, there was no correlation between any copper-chelating capacity of the picolinic acids and their inhibitory effects on the enzyme.

The mechanism of dopamine β-hydroxylase inhibition by these picolinic acids was then investigated. When the inhibitor was added with the substrate or incubated with enzyme before adding the substrate, the inhibitor was uncompetitive for the substrate. The double-reciprocal plot in Fig. 1A characterizes the uncompetitive inhibition of the picolinic acids as typified by Br_2FA. Incubation of the inhibitor with enzyme before the addition of substrate did not influence the inhibitory activity or the

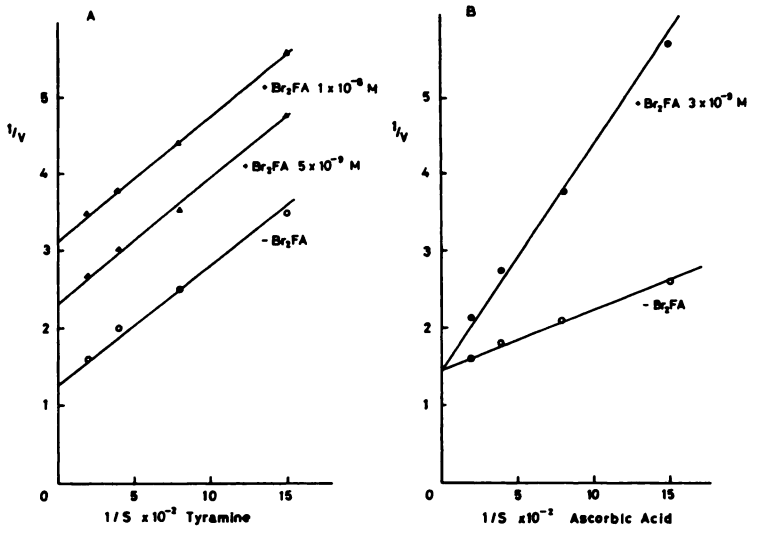


FIG. 1. *Lineweaver-Burk plots of molar tyramine (A) and ascorbic acid (B) concentration against rate of β-hydroxylation of tyramine with and without Br_2FA*

The assay was carried out as described in MATERIALS AND METHODS. The velocities are expressed as A_{330}. O——O, enzyme alone; ▲——▲, with 5 nM Br_2FA; △——△, with 10 nM Br_2FA; ●——●, with 3 nM Br_2FA.

mode of inhibition. The inhibitor was competitive for ascorbic acid, and the double-reciprocal Lineweaver-Burk plot characterizes the competitive inhibition of the compounds as typified by Br_2FA (Fig. 1B). Johnson *et al.* (7) previously noted that the aromatic thioureas were competitive for the substrate but that when the aromatic thioureas were initially incubated for 15 min, increasing concentrations of substrate could not overcome the inhibition. The inhibition of dopamine β-hydroxylase by the picolinic acids was different from the inhibition by the thioureas. Diethyldithiocarbamate was noncompetitive for the substrate and ascorbic acid, and the double-reciprocal Lineweaver-Burk plots for substrate and ascorbic acid were characteristic of noncompetitive inhibition (Fig. 2A and B).

In order to determine whether the picolinic acids are specific inhibitors for dopamine β-hydroxylase, the effects of these compounds on the activity of another copper-containing enzyme, tyrosinase, were studied. The picolinic acids did not inhibit the activity of tyrosinase up to 10 μM, as typified by FA and Br_2FA (Table 3). The concentrations of Br_2FA that produced 50% inhibition of dopamine β-hydroxylase and tyrosinase were 10 nM and 130 μM, respectively, a 10,000-fold difference. The concentration of diethyldithiocarbamate that produced 50% inhibition of dopamine β-hydroxylase and tyrosinase was 1 μM. The inhibitory activities of diethyldithiocarbamate toward both enzymes were identical.

DISCUSSION

Evidence that the mechanism of inhibition of dopamine β-hydroxylase by the picolinic acids is not due primarily to the formation of chelates with the copper of this enzyme were the observations (a) that there was no correlation between any copper-chelating capacity of the picolinic acids and their inhibitory effects on the enzyme, (b) that the addition of copper to the incubation mixture could not overcome the inhibition by the picolinic acids (15), (c) that tyrosinase was not inhibited by these compounds up to a concentration of 10 μM *in vitro* and monoamine oxidase was also not inhibited *in vivo* and *in vitro* (14), (d) that picolinic acid and dipicolinic acid were effective inhibitors of dopamine β-hydroxylase to approximately the same extent in spite of the marked difference

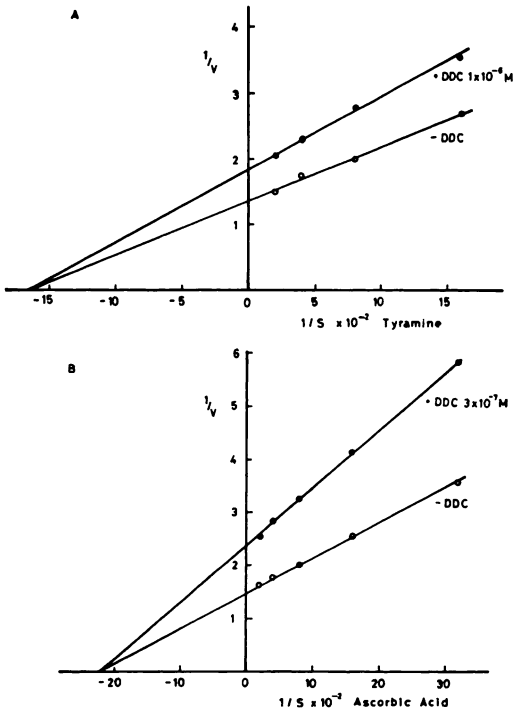


FIG. 2. *Lineweaver-Burk plots of molar tyramine (A) and ascorbic acid (B) concentration against rate of β-hydroxylation of tyramine with and without diethyldithiocarbamate (DDC)*

The assay was carried out as described in MATERIALS AND METHODS. The velocities are expressed as A_{330}. O——O, enzyme alone; ●——●, with 1 μM (A) or 300 nM (B) diethyldithiocarbamate.

between their stability constants for copper (16, 17), and (e) that FA, which is a far less effective ligand of copper than diethyldithiocarbamate, is approximately 13 times more potent an inhibitor of the hydroxylase (Tables 1 and 2). Although many copper-chelating agents are known to inhibit dopamine β-hydroxylase *in vitro* (4), they are probably not specific inhibitors of the enzyme and are less active than Br_2FA. Diethyldithiocarbamate inhibited tyrosinase *in vitro* at approximately the same concentration at which it produced dopamine β-hydroxylase inhibition, but Br_2FA did not inhibit tyrosinase at up to 10,000 times the concentration inhibitory to the hydroxylase. We do not exclude the possibility of chelate formation between the picolinic acids and copper in dopamine β-hydroxylase, because

TABLE 3

Inhibition of tyrosinase by FA, Br_2FA, and diethyldithiocarbamate

Inhibitors were incubated with enzyme for 10 min before adding the substrate. Enzyme activity was determined spectrophotometrically. Details are described in MATERIALS AND METHODS.

Inhibitor	Concentration	Inhibition
	M	%
FA	1×10^{-5}	0
	5×10^{-5}	35
	1×10^{-4}	50
	2×10^{-4}	70
Br_2FA	1×10^{-5}	0
	5×10^{-5}	20
	1×10^{-4}	35
	2×10^{-4}	60
Diethyldithiocarbamate	1×10^{-7}	0
	5×10^{-7}	7
	1×10^{-6}	50
	2×10^{-6}	100

a change in the absorption spectrum of FA was observed at high concentration. Tyrosinase was probably inhibited by chelate formation between the picolinic acids and copper of the enzyme. It should be noted that the most important factor for dopamine β-hydroxylase inhibition is the moiety at position 5 of picolinic acid. This is not directly involved in the chelate formation.

An isotopic study using [^{14}C]dopamine revealed that the conversion of [^{14}C]dopamine to [^{14}C]norepinephrine was significantly inhibited in heart and adrenal glands by injection of the picolinic acids.[3] The reduction of norepinephrine after administration of the picolinic acids seems to be due to inhibition of the hydroxylase *in vivo*.

Our data indicate that the inhibition of dopamine β-hydroxylase by the picolinic acids is not primarily due to their copper chelation. Specificity in the inhibition can be best explained by the steric factors in position 5 of picolinic acids, which affect penetration to the active site. From this point of view, it is reasonable that the substitution

[3] H. Hidaka, N. Takemoto, and K. Takeya, unpublished observations.

of hydrogen for halogen at position 3′ or 4′ of 5-butylpicolinic acid would make the derivatives more potent. It is noteworthy that the most potent inhibitors of dopamine β-hydroxylase, i.e., halofusaric acids (Br_2FA, BrFA, etc.), are more potent antihypertensive agents than FA (18).

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