[Chem. Pharm. Bull. 17(11)2377—2380(1969)]

UDC 577.158.04 : 547.826.09

Inhibition of Dopamine β-Hydroxylase by 5-Alkylpicolinic Acid and Their Hypotensive Effects

Hiroyuki Suda, Tomio Takeuchi,[1a] Toshiharu Nagatsu,[1b] Meiki Matsuzaki, Ikuo Matsumoto,[1c] and Hamao Umezawa[1a]

Institute of Microbial Chemistry,[1a] Department of Biochemistry, Aichi Gakuin University, School of Dentistry,[1b] and Research Laboratories of Banyu Co., Meguro[1c]

(Received May 31, 1969)

As reported by Hidaka, *et al.*,[2] an active compound was found by screening studies of fungus products inhibiting dopamine β-hydroxylase *in vitro* and was identified with fusaric acid, 5-butylpicolinic acid. As described in another paper by Nagatsu, *et al.*,[3] it is a potent inhibitor of dopamine β-hydroxylase *in vitro* and *in vivo*, lowering endogenous levels of norepinephrine and epinephrine in brain, heart, spleen, and adrenal gland.

Homologues of this compound have been synthesized and activities of inhibiting dopamine β-hydroxylase and hypotensive effects have been studied. These results together with kinetic studies on inhibition of the enzyme reaction by 5-pentylpicolinic acid are presented in this paper.

Experimental

Assay of Inhibition of Dopamine β-Hydroxylase by 5-Alkylpicolinic Acids——Dopamine β-hydroxylase was prepared from medullae of beef adrenals. The adrenal medullae were homogenized in 0.02M phosphate buffer of pH 6.5 containing sucrose at 8.5%. The ratio of the buffer to the adrenals was 10:1 in the weight. The homogenized solution was centrifuged at 700*g* for 10 min and the supernatant was centrifuged at 10000*g* for 1 hr. The precipitate was collected and suspended in 0.02M phosphate buffer of pH 6.5 containing sucrose at 8.5%. The weight of the buffer used was the same as that of the adrenals from which the enzyme was extracted. This enzyme solution could be kept more than several months in the frozen state without decrease of the activity. Generally, the enzyme solution was 35 times diluted with 0.02M phosphate buffer of pH 6.5 containing sucrose at 8.5% and 0.1 ml of the diluted solution was incorporated in the reaction mixture. This concentration of the enzyme in the reaction mixture was enough to give the linear progress of the reaction for 30 min. The reaction mixture consisted of the following materials: 1M potassium phosphate buffer of pH 6.5, 0.2 ml; 0.1M ascorbic acid, 0.1 ml; 0.02M fumaric acid in 0.2N NaOH, 0.05 ml; 4 mg/ml of catalase, 0.05 ml; 0.1M tyramine, 0.1 ml; 0.1M N-ethylmaleimide, 0.1 ml; the enzyme solution, 0.1 ml; and the solution of the test material, 0.1 ml, and the total volume was made 1.0 ml with distilled water. After reaction under shaking at 37° for 25 min, 0.2 ml of 50% trichloracetic acid solution was added to cease the reaction and the reaction mixture was passed through a column (5 cm length, 0.6 cm diameter) of Amberlite IR-CG-120 in H^+ form. Distilled water (10.0 ml) was passed through the column and the reaction product (octopamine) which was adsorbed on the column was eluted with 3.0 ml of 4N NH_4OH. The reaction product in the eluate was oxidized to *p*-hydroxy-benzaldehyde by addition of 0.3 ml of 2.0% sodium periodate and, after 6 min, 0.3 ml of 1.0% sodium metabisulfite was added. The optical density at 330 mμ was determined.

Reaction Mixture of the Kinetic Studies——It consisted of the following materials: 1M potassium phosphate buffer of pH 5.5, 0.2 ml; 0.1M ascorbic acid, 0.1 ml; 0.02M fumaric acid in 0.2N NaOH, 0.1 ml; 0.4 mg/ml

1) Location: *a) 14–23, Kamiosaki 3-chome, Shinagawa-ku, Tokyo; b) 2–11, Suemori-dori, Chikusa-ku, Nagoya; c) 9–3, Shimomeguro 2-chome, Meguro-ku, Tokyo.*

2) H. Hidaka, T. Nagatsu, K. Takeya, T. Takeuchi, H. Suda, K. Kojiri, M. Matsuzaki, and H. Umezawa, *J. Antibiotics* "in press" (1969).

3) T. Nagatsu, H. Hidaka, H. Kuzuya, K. Takeya, H. Umezawa, T. Takeuchi, and H. Suda, *Biochem. Pharmacol.*, in press.

of catalase, 0.1 ml; 0.1M tyramine, 0.1 ml; 0.1M N-ethylmaleimide, 0.1 ml; the enzyme solution, 0.1 ml; the solution of the test material 0.1 ml, and the total volume was made 1.0 ml with distilled water. This reaction mixture was preincubated for 2 min at 39° and the reaction was started by adding the substrate. The reaction mixture was incubated for 30 min at 39°.

Toxicity and the Hypotensive Effect——All materials were dissolved in distilled water and pH was adjusted to 7.0. LD_{50} in mice was examined using 60 mice divided into 6 groups for each compound. The mice were observed until 10 days after the injection. LD_{50} value was calculated by means of Behrens–Kärber. 50 mg/kg of each compound was intraperitoneally injected to 4 rabbits anesthetized with pentobarbital-Na (30 mg/kg *i.p.*). The blood pressure was measured with a mercury manometer inserting a cannula into the carotid artery at 5, 15, 30 min, 1, 2, 4, 6 and 24 hr after the injection.

Preparation of 5-Alkylpicolinic Acid——5-Methylpicolinic acid[4] was prepared by selenium dioxide oxidation of 2-methyl-5-methylpyridine and recrystallized from ethanol–benzene, mp 164—166°. 5-Ethylpicolinic acid[4] was prepared by the similar process and recrystallized from ethyl acetate–hexane, mp 103—106°. 2-Methylpyridines containing 5-alkyl groups higher than ethyl were prepared from 2-methyl-5-cyanopyridine with Grignard reagentscontaining the corresponding alkyl groups followed by reduction with hydrazine and potassium hydroxide. They were oxidized to the corresponding 5-alkylpicolinic acids with selenium dioxide. The melting points of 5-alkylpicolinic acids containing 5-alkyl group higher than ethyl were as follows: 5-propylpicolinic acid recrystallized from ethyl acetate–hexane, mp 126—127°; 5-butylpicolinic acid (fusaric acid)[5] recrystallized from ethyl acetate–hexane, mp 99—100°; 5-pentylpicolinic acid[5] recrystallized from isopropyl ether, mp 104—105°; 5-hexylpicolinic acid recrystallized from isopropyl ether, mp 101—102°; 5-heptylpicolinic acid recrystallized from ethyl ether-petroleum ether, mp 109—110°, 5-octylpicolinic acid recrystallized from ethyl ether-petroleum ether, mp 105—106°; 5-nonylpicolinic acid recrystallized from ethyl ether-petroleum ether, mp 110—111°. The last four 5-alkylpicolinic acids are new compounds which were synthesized for the first time.

Table I. Effect on the Blood Pressure in the Rabbits under Pentobarbital-Na Anesthesia (30 mg/kg *i.p.*) by 5-Alkylpicolinic Acids, and Their Toxicity in Mice

5-Alkyl chain	Body weight, kg (Range)		Blood pressure and depression %, B.P. ± S.D. Time after administration 0 min	5	15	30	1 hr	2	4	6	24 hr	Toxicity in mice, LD_{50} mg/kg (*i.p.*)
–H	(2.0—2.3)	B.P.	115± 9	120	115	113	112± 7	109± 8	109± 8	109± 7	113	360
		%		−4	0	2	3	14	14	14	2	
$-CH_3$	(2.4—2.6)	B.P.	96± 4	86	86	80	77±10	77± 9	79±11	79±11	89	175
		%		10	10	17	20	20	18	18	7	
$-C_2H_5$	(2.1—2.5)	B.P.	113± 7	108	105	99	83± 9	83±10	94±11	96± 9	115	125
		%		4	7	12	27	27	17	15	−2	
$-C_3H_7$	(2.4—2.6)	B.P.	92± 6	88	81	77	70± 5	60±12	74± 7	80± 7	—	120
		%		4.3	12	16	24	35	20	13	—	
$-C_4H_9$	(2.5—2.5)	B.P.	103± 7	98	—	84	80± 6	71± 7	70± 9	67± 7	96	80
		%		5	—	18	22	31	32	35	7	
$-C_5H_{11}$	(2.3—2.6)	B.P.	98±11	100	86	75	69±11	59± 4	57± 6	69± 8	72	70
		%		−2	12	23	30	40	42	30	27	
$-C_6H_{13}$	(2.3—2.6)	B.P.	95± 6	100	83	81	75± 7	74± 7	85±10	93± 5	—	85
		%		−5	13	15	21	22	11	2	—	
$-C_7H_{15}$	(2.4—2.7)	B.P.	83± 5	104	79	71	64± 8	50±12	46±10	47±10	80	45
		%		−25	4.8	15	23	40	44	43	3.6	
$-C_8H_{17}$	(2.4—2.7)	B.P.	96± 7	98	90	72	61± 8	87±10	99±12	—	—	62
		%		−2.1	6.3	25	37	9.4	−3.1	—	—	
$-C_9H_{19}$	(2.4—2.6)	B.P.	93± 7	86	87	81	83±12	92±10	101±14	—	—	75
		%		7.5	6.5	12	11	1.1	−8.6	—	—	

B.P.: blood pressure, mmHg %: depression %
Each figure is average of 4 rabbits following intraperitoneal administration of 50 mg/kg single doses.

4) D. Jerchel and J. Heider, *Ann. Chem.*, **613**, 153 (1968).
5) E.R. Ebersole, C. Guttentag, and P.W. Wilson, *Arch. Biochem. Biophys.*, **3**, 399 (1943).

Results and Discussion

Activities of Inhibiting Dopamine β-Hydroxylase, the Hypotensive Effects and LD_{50} to Mice

The molar concentrations of each 5-alkylpicolinic acid for 50% inhibition of the enzyme reaction are shown in Fig. 1. Picolinic acid and all 5-alkylpicolinic acids inhibit dopamine β-hydroxylase reaction. The activities are dependent on the number of carbon atoms in 5-alkyl group and 5-butyl and 5-pentyl compounds show the stronger inhibition than the others. The hypotensive effects of 5-alkylpicolinic acids are shown in Table I. Increasing carbon

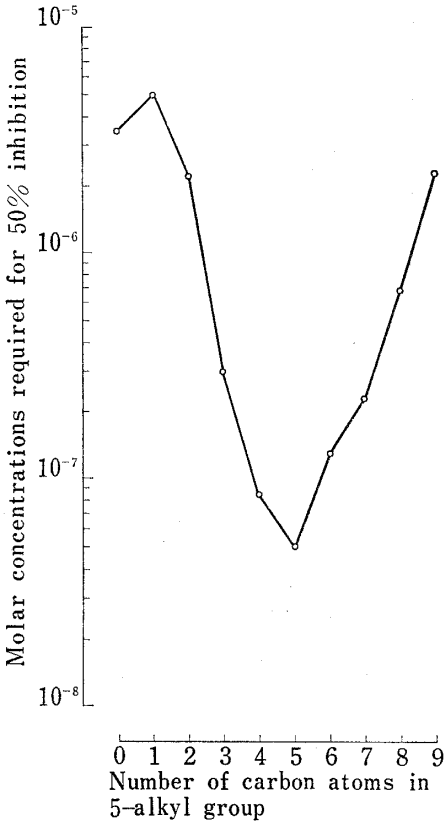

Fig. 1. Concentrations of 5-Alkylpicolinic Acids for 50% Inhibition of Dopamine β-Hydroxylase

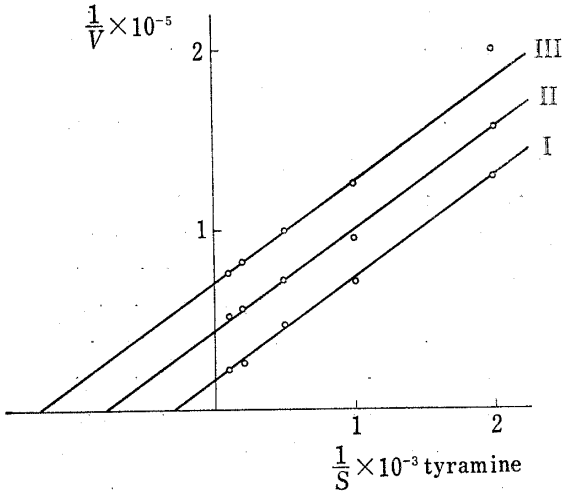

Fig. 2. Double Reciprocal Plots of Tyramine Concentration *vs.* Rate of Tyramine Hydroxylation, with and without 5-Pentylpicolinic Acid

I: tyramine
II: tyramine plus 5-pentylpicolinic acid 1.0×10^{-7}M
III: tyramine plus 5-pentylpicolinic acid 2.5×10^{-7}M
Activity was measured with the purified adrenal enzyme under the conditions described in the text. Tyramine was varied between 1×10^{-3}M and 5×10^{-4}M.

numbers in 5-alkyl group up to 4 or 5, the hypotensive effect becomes stronger, and 5-butyl- and 5-pentyl-picolinic acids show the stronger hypotensive effect than 5-propionyl-, 5-ethyl- and 5-methyl-picolinic acid. Conforming with the effect on dopamine β-hydroxylase, 5-hexylpicolinic acid shows weaker hypotensive effect than 5-butyl or 5-pentyl compounds. However, 5-heptyl and 5-octyl compounds show the stronger hypotensive effects than 5-hexylpicolinic acid. The hypotensive effects of 5-heptyl and 5-octyl compounds may be due to their toxicities. LD_{50} values of all compounds also shown in Table I. In the test of the hypotensive effect, 50 mg/kg of each compound was intraperitoneally injected and therefore, the doses of heptyl, octyl and nonyl compounds are closed to their LD_{50}.

Nagatsu, *et al.*[3] reported that 5-butylpicolinic acid showed inhibition of dopamine β-hydroxylase *in vivo*, lowering levels of norepinephrine and epinephrine markedly.

Kinetic Studies of 5-Pentylpicolinic Acid on Dopamine β-Hydroxylase

The results studying the relation of 5-pentylpicolinic acid to the substrate (tyramine) are expressed in double reciprocal plots and shown in Fig. 2. It is interesting that inhibition

of dopamine β-hydroxylase by 5-pentylpicolinic acid is of the uncompetitive type, implying that this compound binds with the enzyme-substrate complex. Nagatsu, *et al.*[3] also observed that 5-butylpicolinic acid showed the uncompetitive type of inhibition with the substrate. The results of testing the relation of the inhibitor to the cofactor (ascorbic acid) are shown in Fig. 3. As shown in Fig. 3 5-pentylpicolinic acid shows competitive inhibition with ascorbic acid.

As reported by Friedman and Kaufman,[6] dopamine β-hydroxylase contains copper, and during the hydroxylation reaction, the copper is reduced from Cu^{2+} to Cu^{+} by ascorbic acid. On exposure of the reduced enzyme to substrate, a large part of the Cu^{+} is reoxidised to Cu^{2+} and approximately equivalent amount of hydroxylated products are formed. 5-Alkylpicolinic acid has a property to chelate with cupric ion. These observation suggests that, during hydroxylation reaction, 5-alkylpicolinic acid compete with ascorbic acid, chelating with copper. Nicotinic acid which does not chelate with copper showed no inhibition on dopamine β-hydroxylase. Therefore, this chelation of 5-alkylpicolinic acid with copper in the enzyme seems to be its primary site of action on dopamine β-hydroxylase.

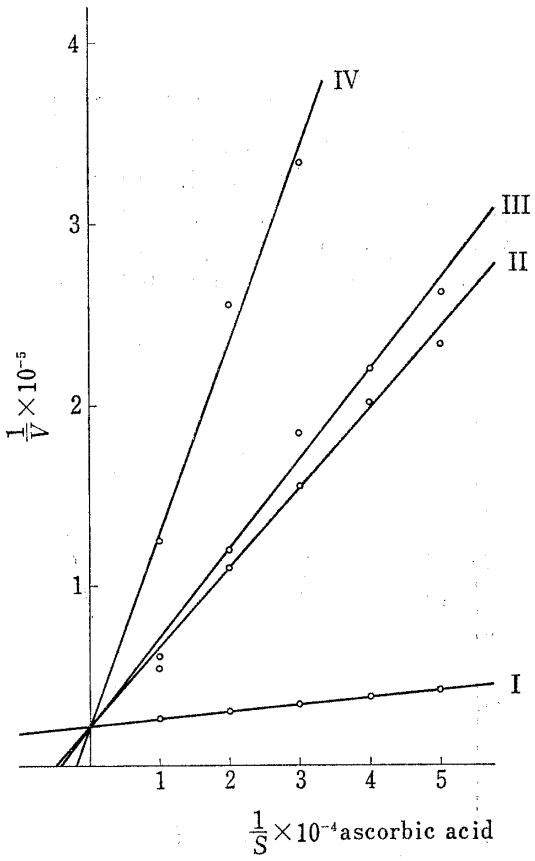

Fig. 3. Double Reciprocal Plots of Ascorbic Acid Concentrations *vs.* Rate of Tyramine Hydroxylation, with and without 5-Pentylpicolinic Acid

I: ascorbic acid
II: ascorbic acid plus 5-pentylpicolinic acid 8.0×10^{-8}M
III: ascorbic acid plus 5-pentylpicolinic acid 1.0×10^{-7}M
IV: ascorbic acid plus 5-pentylpicolinic acid 2.5×10^{-7}M
Activity was measured with the purified adrenal enzyme under the conditions described in the text. Ascorbic acid was varied between 1×10^{-2}M and 2×10^{-3}M.

6) S. Friedman and S. Kaufman, *J. Biol. Chem.*, **240**, 4763 (1965).