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Aromatic Amino Acid Hydroxylase Inhibitors. 3.† *In Vitro* Inhibition by Azadopamine Analogs

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In the present study a series of azadopamines (1-aminoethyl-5-hydroxy-4-pyridones) was synthesized in anticipation that they would serve as inhibitors of tyrosine hydroxylase, the enzyme catalyzing the rate-limiting reaction in the biosynthesis of norepinephrine. The azadopamines, synthesized through a route involving diamine condensation with kojic acid, were found to inhibit bovine adrenal tyrosine hydroxylase, rat liver phenylalanine hydroxylase, and rat brainstem tryptophan hydroxylase. Further inhibition studies revealed uncompetitive kinetics with respect to substrate, noncompetitive kinetics with respect to reduced pteridine cofactor, and prevention of inhibition by Fe^{2+}. The studies reported in this paper lead us to the conclusion that hydroxylase inhibition by the azadopamines is partially but not entirely achieved through a Fe^{2+} chelation mechanism.

One approach to antihypertensive drugs has been to synthesize agents which inhibit the biosynthesis of norepinephrine (NE). Although several enzymatic steps are available for pharmacological control, the demonstration by Udenfriend and coworkers[1] that tyrosine hydroxylase (TH) represents the rate-limiting step in NE biosynthesis has stimulated interest in inhibitors of this enzyme.

The finding that most catechols, including NE, inhibit TH *in vitro* suggested that a feedback or end-product inhibition may be responsible for the overall regulation of NE synthesis.[2,3] Costa and Neff[4] have reviewed the evidence compatible with such a feedback process. In addition, recent evidence has accumulated to indicate that catecholamines can regulate their own synthesis by feedback repression as well.[5]

To date, most studies dealing with the synthesis of specific TH inhibitors have involved preparation of substrate analogs[6-10]—the "classical" antimetabolite approach. However, catecholamines and other catechols inhibit TH in another manner; *i.e.*, they are noncompetitive with respect to substrate and competitive with respect to the tetrahydropteridine cofactor (PtH_4). Such inhibition of TH by catecholamines could represent a mechanism of feedback control of catecholamine biosynthesis. Structural analogs of catecholamines containing a 3-hydroxy-4-pyridone ring system have now been synthesized in the hope that such compounds would effectively inhibit aromatic amino acid hydroxylases both *in vitro* and *in vivo*.

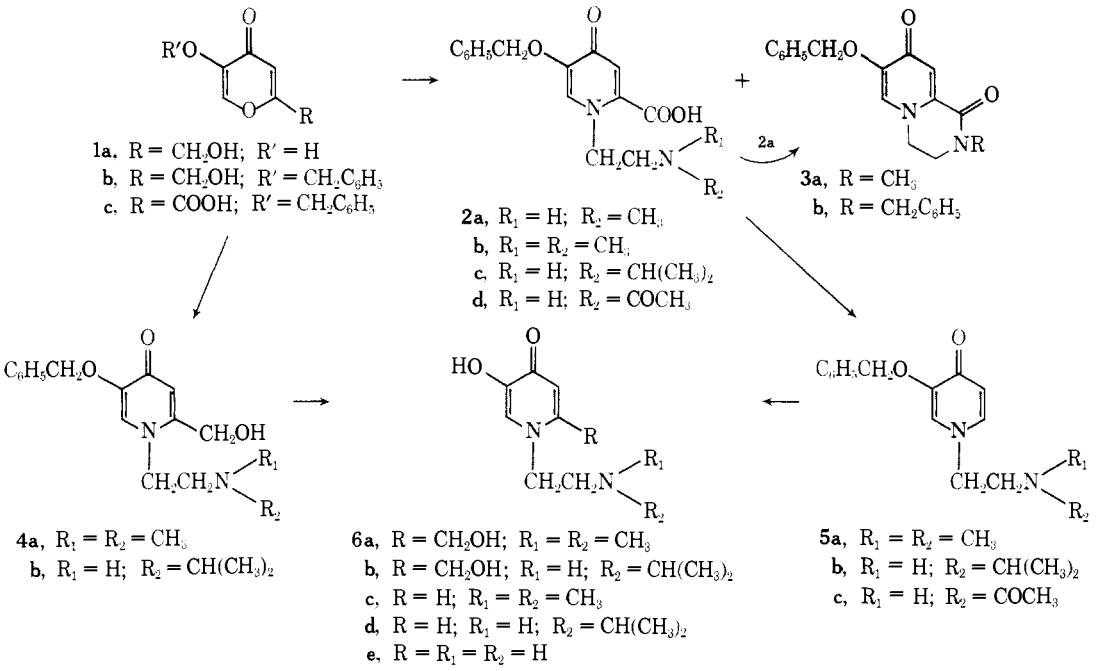
† This manuscript is dedicated to Dr. Alfred Burger as a tribute to his leadership in fostering the field of medicinal chemistry. His own research on agents which modify the actions of neurotransmitters is well known, and we hope this paper assumes the high standards he helped to establish.

‡ Taken in part from the dissertation presented by Larry E. Hare, June 1971, to the Graduate School of the University of Michigan in partial fulfillment of the requirement for the Doctor of Philosophy degree.

Since a convenient route to 4-pyridones is condensation of 4-pyrones with ammonia or primary amines,[11] commercially available kojic acid (**1a**) was selected as starting material for the synthesis of a series of azadopamine analogs. Condensation of the benzyl ether of kojic acid (**1b**) with the appropriate diamine afforded the desired pyridones **4a,b.** Difficulties with the Jones oxidation of the hydroxymethyl group of **4a** necessitated conversion to the pyronecarboxylic acid **1a** prior to the insertion reaction. Thus, when **1c** was treated with *N,N*-dimethyl-, *N*-isopropyl-, or *N*-acetoxyethylenediamine, the desired amino acids **2b–d** were the only products isolated. When the insertion reaction was performed with *N*-methyl- and *N*-benzylethylenediamine, however, the major products isolated were the corresponding bicyclic lactams **3a,b.** Moreover, **2a** could be converted to **3a** by heating in diphenyl ether. Similar heat treatment of **2b–d,** on the other hand, caused thermal decarboxylation to afford **5a–c.** Acid-catalyzed removal of the benzyl-protecting group furnished the desired azadopamines **6a–e** whose physical properties are recorded in Table I. Susceptibility of the free bases to air oxidation necessitated preparation of the corresponding hydrochloride salts for storage and biological testing.

Enzyme Studies. The three synthetic azadopamines (**6c–e**) were studied as inhibitors of the aromatic amino acid hydroxylases. The results of the inhibition studies are given in Table II. The aza analogs are comparable in activity to dopamine (DA) and NE, which are well-documented inhibitors of the aromatic hydroxylase enzymes.[12-14]

Attempts were made to determine the mode of hydroxylase inhibition of the azadopamines. In a recent review on tyrosine hydroxylase inhibitors, three classes of inhibitors are described: those which are competitive with substrate; those which are competitive with PtH_4; and diva-



lent metal chelating agents which complex with Fe^{2+}.[10] Amino acids such as α-methyltyrosine and 3-iodotyrosine are substrate inhibitors.[2,12] Bublitz[14] has recently demonstrated the *in vitro* inhibition of PH by catecholamines in competition with PtH_4; he also observed a second mechanism for PH inhibition which involves the inactivation of the enzyme by unstable *o*-quinones arising from the catechols. Compounds of widely differing structure have been demonstrated to inhibit TH through Fe^{2+} chelation.[15,16]

Double reciprocal substrate-velocity plots (Figures 1 and 2) revealed that azadopamine (**6e**) was uncompetitive with phenylalanine (PH from rat liver) and noncompetitive with PtH_4. Similar results were obtained employing bovine adrenal TH.

Table III shows the effects of Fe^{2+} concentration on PH inhibition. Dimethylazadopamine (**6c**) inhibition of PH is almost completely reversed by supplementation with Fe^{2+}. In contrast, inhibitions by DA and NE are almost completely unaffected by Fe^{2+} concentration. If Fe^{2+} chelation with the 3-hydroxy-4-pyridone moiety is the mode of inhibition of **6c**, then protection of the phenolic hydroxyl with a benzyloxy group might very well prevent such inhibition. In this light it is interesting that **5b** is only a weak inhibitor of the hydroxylases. Furthermore, kojic acid (**1a**) which contains the 3-hydroxy-4-pyrone moiety inhibits PH but to a much lesser extent than the azadopamines. Apparently the inhibition by kojic acid is due solely to Fe^{2+} chelation as supplements of Fe^{2+} prevent the inhibition (Table III).

The remaining question in this investigation was whether the relative potency of the azadopamines as hydroxylase inhibitors could be directly correlated with their chelating abilities. Polarographic experiments (Table IV) revealed that the substituted azadopamines, **6c** and **6d**, are definitely better Fe^{2+} chelators than **6e**. These data correlate very well with their activities as hydroxylase inhibitors. At the same concentrations DA and NE did not chelate Fe^{2+}. Curiously though, kojic acid is a better chelator than either **6c** or **6d**, while the latter compounds are superior as hydroxylase inhibitors. Perhaps the mode of inhibition of the azadopamines is due only in part to Fe^{2+} chelation.

In vitro pharmacological tests (Dr. P. N. Patil, The Ohio State University) have shown that the azadopamine analog **6e** was essentially devoid of adrenergic activity on guinea-pig atria at a concentration of 3×10^{-4} *M*. On isolated rabbit aorta, a slight contraction was observed at 3×10^{-4} *M* and the order of activity was approximately $1/10{,}000$th that of (−)-norepinephrine. In another experiment, there was no blockade of (−)-norepinephrine in the presence of 3×10^{-4} *M* **6e**.

Experimental Section§

5-Benzyloxy-2-hydroxymethyl-4-pyrone (1b). Anhydrous K_2CO_3 (276.2 g, 2 mol) was suspended in a solution of kojic acid (**1a**) (142 g, 1 mol) and benzyl chloride (253.2 g, 2 mol) in DMF (700 ml). The temperature of the reaction mixture was raised to 100–120° and maintained for 3 hr. The dark reaction mixture was allowed to cool, poured into H_2O (1000 ml), and extracted with $CHCl_3$. The organic phase was washed with H_2O, dried ($MgSO_4$), and evaporated. Recrystallization of the resulting tan solid from $CHCl_3$ provided **1b** as white needles (123.0 g, 53%): mp 128–130° (lit.[17] mp 131–133°).

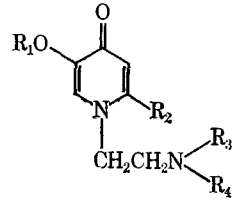
5-Benzyloxy-4-pyrone-2-carboxylic Acid (1c). Compound **1b** (10.0 g, 43.2 mmol) was dissolved in acetone (500 ml), cooled in an ice bath, and titrated with Jones reagent (25 ml). The inorganic material was removed by filtration and the filtrate evaporated to dryness. Pure **1c** (8.2 g, 77%) was obtained by recrystallization from MeOH: mp 195–197° (lit.[18] mp 196°).

5-Benzyloxy-1-(2′-methylaminoethyl)-4-pyridone-2-carboxylic Acid (2a). *N*-Methylethylenediamine (15 ml) was added to compound **1c** (8.0 g, 32.4 mmol). An exothermic reaction occurred and the solution became orange in color. The reaction mixture was warmed slightly on a steam bath to dissolve all the starting material. The solution was stirred at room temperature 4 hr and evaporated to dryness. A solid crystallized from MeOH–Et_2O. Fractional recrystallization from MeOH afforded two products. The desired product **2a** (0.63 g, 6%) was insoluble in MeOH. An analytical sample was recrystallized from H_2O: mp 195–197°; ir and nmr as expected. *Anal.* ($C_{16}H_{18}N_2O_4 \cdot 0.5H_2O$) C, H. The byproduct (4.9 g, 53%) was recrystallized from MeOH and CH_3CN and was characterized as **3a** below.

7-Benzyloxy-1,8-dioxo-2-methyl-3,4-dihydroxy-2*H*-pyrido[1,2-*a*]pyrazine (3a). An analytical sample was recrystallized from

§ The nmr spectra were obtained with a Varian A-60A spectrometer. Infrared spectra were recorded on a Perkin-Elmer 337 spectrophotometer. Mass spectra were recorded on a Du Pont 21-490 mass spectrometer. Colorimetric determinations (enzyme studies) were made on a Beckman DB-GT spectrophotometer. Polarographic spectra were recorded on a Leeds and Northrup 62200 recording polarograph. Radioactivity was determined on a Beckman LS-150 liquid scintillation system. The melting points were measured on a Thomas-Hoover apparatus and are corrected. Where analyses are indicated only by symbols of the elements, analytical results obtained for those elements were within ±0.4% of the theoretical values. Analyses were performed by Spang Microanalytical Laboratory, Ann Arbor, Mich., and Midwest Microlab, Ltd., Indianapolis, Ind.

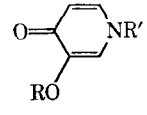
Table I. Azadopamines



No.	R_1	R_2	R_3	R_4	Method	Crystn solvent	Mp, °C	Yield, %	Formula	Analyses
5a	$CH_2C_6H_5$	H	CH_3	CH_3	A	EtOH	192–193	74	$C_{16}H_{20}N_2O_2 \cdot 2HCl$	C, H
5b	$CH_2C_6H_5$	H	H	$CH(CH_3)_2$	A	EtOH	170–172	75.5	$C_{17}H_{20}N_2O_2 \cdot 2HCl \cdot H_2O$	C, H, Cl
5c	$CH_2C_6H_5$	H	H	$COCH_3$	A	$CHCl_3$–hexane	166–166.5	48.5	$C_{16}H_{18}N_2O_3 \cdot H_2O$[a]	C, H
6a	H	CH_2OH	CH_3	CH_3	B	MeOH	195–197	93	$C_{10}H_{16}N_2O_3$	C, H
6b	H	CH_2OH	H	$CH(CH_3)_2$	C	EtOH	218–221	74	$C_{11}H_{17}N_2O_3 \cdot 2HCl$	H, Cl; C[b]
6c	H	H	CH_3	CH_3	B	CH_3CN–Et_2O	165–166	95	$C_9H_{14}N_2O_2$	C, H
6d	H	H	H	$CH(CH_3)_2$	C	EtOH	225–227	89	$C_{10}H_{16}N_2O_2 \cdot 2HCl$	H; C[c]
6e	H	H	H	H	C	EtOH–H_2O	265 dec	90	$C_7H_{10}N_2O_2 \cdot HCl$	C, H, N, Cl

[a]Mass spectrum, *m/e* 286 (M). [b]C: calcd, 44.16; found, 44.67. [c]C: calcd, 44.62; found, 44.16.

Table II. Inhibition of Aromatic Hydroxylase Enzymes[a]



Compd	R	R′	Per cent inhibition: Phenylalanine hydroxylase[b] 1.0 m*M*	Phenylalanine hydroxylase[b] 0.1 m*M*	Tyrosine hydroxylase[c] 1.0 m*M*	Tyrosine hydroxylase[c] 0.1 m*M*	Tryptophan hydroxylase[d] 1.0 m*M*	Tryptophan hydroxylase[d] 0.1 m*M*
6e	H	$CH_2CH_2NH_2$	58.3 ± 2.8	7.7 ± 1.6	66.1 ± 1.0	20.2 ± 2.3	35.0 ± 3.6	9.0 ± 2.3
6d	H	$CH_2CH_2NHCH(CH_3)_2$	93.9 ± 1.3	37.8 ± 5.1	89.9; 97.0	43.5 ± 0.7	55.2 ± 2.7	22.8 ± 0.3
6c	H	$CH_2CH_2N(CH_3)_2$	97.0 ± 2.6	64.1 ± 3.0	87.8 ± 1.5	50.6 ± 9.6	45.9 ± 2.6	22.8 ± 4.3
5b	$C_6H_5CH_2$	$CH_2CH_2N(CH_3)_2$	3.8; 3.1	0.8; 0.0	25.6 ± 2.3	8.7 ± 1.2		
L-Norepinephrine			58.6 ± 2.5	18.2 ± 3.2	45.6 ± 3.2	23.9; 19.0		
Dopamine			78.4 ± 4.5	30.7 ± 2.0	72.4 ± 0.3	41.2; 40.5		
Kojic acid			27.7 ± 1.5	2.1; 0.0	23.4 ± 6.0	0.1 ± 0.0		

[a]For details of these assays see the Experimental Section. [b]Assay concentration: phenylalanine = 1.0 m*M*; PtH_4 ($AHDMPH_4$) = 1.0 m*M*. [c]Assay concentration: tyrosine = 0.05 m*M*; PtH_4 ($DMPH_4$) = 2.3 m*M*. [d]Assay concentration: tryptophan = 0.01 m*M*.

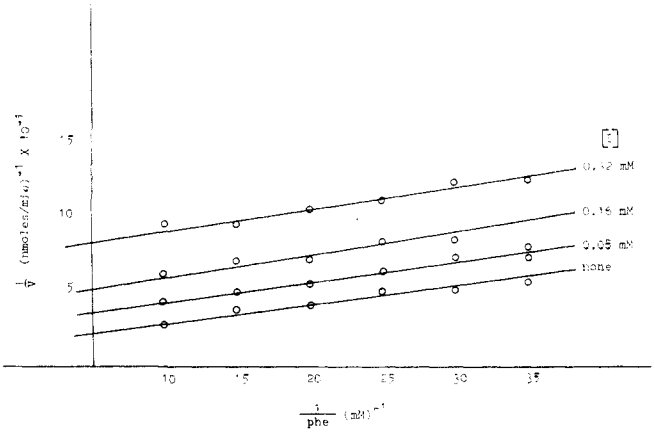


Figure 1. Inhibition of phenylalanine hydroxylase by azadopamine employing phenylalanine as the variable substrate. The concentration of PtH_4 = 0.1 m*M*. For details of the assay see the Experimental Section.

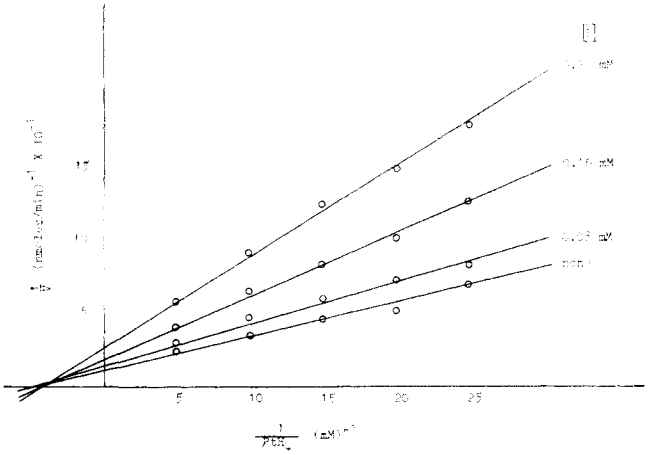


Figure 2. Inhibition of phenylalanine hydroxylase by azadopamine employing PtH_4 as the variable substrate. The concentration of phenylalanine = 1.0 m*M*. For details of the assay see the Experimental Section.

Table III. Effects of Fe^{2+} Concentration on Inhibition of Phenylalanine Hydroxylase[a,b]

Inhibitor	Concn, m*M*	Fe^{2+} concn, m*M*[c]	% inhibition
Dimethylazadopamine	0.08	0.00	55.2
	0.08	0.04	30.8
	0.08	0.08	12.1
	0.08	0.50	4.9
Dopamine	0.10	0.00	26.7
	0.10	0.04	31.6
	0.10	0.08	31.4
	0.10	0.50	23.2
Kojic acid	1.00	0.00	36.7
	1.00	0.04	18.3
	1.00	0.08	3.5
	1.00	0.50	5.7

[a]The preparation and assay of phenylalanine hydroxylase is described in the Experimental Section. [b]Concentration of phenylalanine = 1.0 m*M*; concentration of PtH_4 (AHD-MPH_4) = 0.10 m*M*. [c]Added as $FeSO_4$.

CH_3CN: mp 196–197°; ir (KBr) 1675 [C(=O)N] and 1620 cm^{-1} (C=O); nmr (CF_3CO_2H-$CDCl_3$) δ 8.20 [s, 1 H, HC(N)=C], 8.07 [s, 1 H, C(=O)C(N)=CHC(=O)], 7.43 (s, 5 H, Ar), 5.32 (s, 2 H, $ArCH_2O$), 4.73 [t, 2 H, $NCH_2CH_2NC(=O)$], 3.95 [t, 2 H, $NCH_2CH_2NC(=O)$], and 3.27 (s, 3 H, NCH_3). *Anal.* ($C_{16}H_{16}N_2O_3$) C, H.

5-Benzyloxy-1-(2′-dimethylaminoethyl)-4-pyridone-2-carboxylic Acid (2b). Compound 1c (10.0 g, 40 mmol) was dissolved in *N,N*-dimethylethylenediamine (15 ml) by heating. The temperature was maintained at 75° for 4 hr. The reaction mixture was evaporated to dryness and crystallized from $CHCl_3$–petroleum ether. The resulting yellow powder was washed with Et_2O and recrystallized from MeOH–Et_2O, providing **2b** (6.7 g, 53%): mp 98–100°; ir (KBr) 2900–2100 (NH_3^+), 1625 cm^{-1} (COO^-); nmr (CF_3CO_2H-$CDCl_3$) δ 8.38 [s, 1 H, *H*C(N)=C], 8.06 [s, 1 H, C(N)=CH), 7.44 (s, 5 H, Ar), 5.33–5.13 (m, 4 H, $ArCH_2O$, NCH_2CH_2N), 3.87 (t, 2 H, NCH_2CH_2N), 3.13 and 3.06 [2 s, 6 H, $N(CH_3)_2$]. Compound **2b** was used in subsequent reactions without further purification.

5-Benzyloxy-1-(2′-isopropylaminoethyl)-4-pyridone-2-carboxylic Acid (2c). Compound 1c (12.3 g, 50 mmol) *N*-isopropylethylenediamine (15.3 g, 150 mmol) in CH_3CN (200 ml) was heated at 70–80° for 2 hr. The mixture was chilled in an ice bath and the resulting precipitate was collected. Recrystallization from Me_2CO–MeOH gave pure 2c (46.6%): mp 220–221°; ir and nmr as expected. *Anal.* ($C_{18}H_{22}N_2O_4$) C, H.

5-Benzyloxy-1-(2′-acetylaminoethyl)-4-pyridone-2-carboxylic Acid (2d). Compound 1c (9.9 g, 40 mmol) *N*-acetylethylenediamine (12.2 g, 120 mmol) in CH_3CN (200 ml) and dioxane (50 ml) was heated under reflux for 8 hr. The reaction mixture was allowed to cool to room temperature and the precipitate was collected by filtration. The precipitate was washed with CH_3CN and recrystallized from absolute EtOH to give pure **2d** (12.5 g, 95.5%): mp 179–181°; ir and nmr as expected. *Anal.* ($C_{17}H_{18}N_2O_5 \cdot H_2O$) C, H.

7-Benzyloxy-1,8-dioxo-2-benzyl-3,4-dihydro-2*H*-pyrido[1,2-*a*]pyrazine (3b). Compound 1c (4.92 g, 20 mmol) *N*-benzylethylenediamine (11.5 g, 78 mmol) in CH_3CN (100 ml) was heated under reflux for 8 hr. The reaction mixture was concentrated to half its original volume and allowed to cool to room temperature. The white precipitate which separated was collected by filtration, washed with Me_2CO, and recrystallized from CH_3CN–MeOH to give pure **3b** (3.2 g, 42.5%): mp 227–228°; ir and nmr as expected. *Anal.* ($C_{22}H_{20}N_2O_3$) C, H.

5-Benzyloxy-1-(2′-dimethylaminoethyl)-2-hydroxymethyl-4-pyridone (4a). A suspension of 1b (5.0 g, 21.6 mmol) in *N,N*-dimethylethylenediamine (5 ml) was heated at 60–70° for 2 hr during which time the solid dissolved. The solution was evaporated to dryness and the residue was triturated in petroleum ether to give **4a** as a yellow powder (4.6 g, 71%). An analytical sample was recrystallized from MeOH: mp 106–107°; ir and nmr as expected. *Anal.* ($C_{17}H_{22}N_2O_3$) C, H.

5-Benzyloxy-1-(2′-isopropylaminoethyl)-2-hydroxymethyl-4-pyridone (4b). A suspension of 1b (4.46 g, 20 mmol) in *N*-isopropylethylenediamine (5.1 g, 50 mmol) was heated at 75° for 2 hr during which time the solid dissolved and the reaction mixture became reddish brown. The excess amount of amine was removed under reduced pressure and the residue was triturated with petroleum ether. Attempts to solidify the oily residue failed to give any crystalline products. The crude residue was then chromatographed on a silica gel column (4 × 30 cm) and eluted with MeOH–$CHCl_3$ (1:19) to give pure 4b as a colorless oil (1.14 g, 18.5%). A portion of this oil was used to prepare the HCl salt: mp 181–183°; ir and nmr as expected. *Anal.* ($C_{18}H_{24}N_2O_3 \cdot 2HCl \cdot 0.5H_2O$) C, H, Cl.

Attempted Decarboxylation of 5-Benzyloxy-1-(2′-methylaminoethyl)-4-pyridone-2-carboxylic Acid (2a). Compound 2a (0.3 g, 1.0 mmol) in diphenyl ether (5 ml) was heated at 200° under N_2 for 1 hr. The solution was diluted with $CHCl_3$ and extracted with 10% HCl. A precipitate formed in the aqueous layer. The suspension was neutralized (10% Na_2CO_3) and the solid (0.2 g, 72%) was collected by filtration. Recrystallization from CH_3CN afforded a white solid (mp 196–197°) which was spectroscopically identical with an authentic sample of 3a.

5-Benzyloxy-1-(2′-dimethylaminoethyl)-4-pyridone (5a). General Method A. Compound 2b (2.0 g, 6.3 mmol) was dissolved in diphenyl ether (10 ml) by heating at 190°. The evolution

Table IV. Polarographic Determination of Fe^{2+} Chelation[a]

Compound	% Fe^{2+} chelated
6e, azadopamine	16
6d, isopropylazadopamine	24
6c, dimethylazadopamine	35
1a, kojic acid	42
Dopamine	0

[a]For details of these determinations see the Experimental Section.

of CO_2 was complete in 1 hr. After the reaction mixture had cooled, it was diluted with Et_2O and extracted with 10% HCl. The aqueous phase was neutralized with 10% NaOH and the alkaline solution evaporated to dryness. The residue was washed with $CHCl_3$ and evaporated to give **5a** as a yellow oil (1.3 g, 74%). An analytical sample of the HCl salt was prepared by treatment of a $CHCl_3$ solution of the oil with *i*-PrOH–HCl. The resulting white crystals were recrystallized from EtOH: mp 192–193°; ir and nmr as expected. *Anal.* ($C_{16}H_{20}N_2O_2 \cdot 2HCl$) C, H.

1-(2′-Dimethylaminoethyl)-5-hydroxy-2-hydroxymethyl-4-pyridone (6a). General Method B. Compound **4a** (2.0 g, 6.6 mmol) was hydrogenated in MeOH (20 ml) over 5% Pd/C (0.3 g) at 43 psi and room temperature for 5 hr. The catalyst was removed by filtration and compound **6a** (1.3 g, 93%) crystallized from the filtrate. An analytical sample was recrystallized from MeOH: mp 195–197°; positive $FeCl_3$ test; ir and nmr as expected. *Anal.* ($C_{10}H_{16}N_2O_3$) C, H.

1-Aminoethyl-5-hydroxy-4-pyridone (Azadopamine, 6e). General Method C. Compound **5c** (1.44 g, 5 mmol) in 20% aqueous HCl (50 ml) was heated under reflux for 20 hr. The reaction mixture was then evaporated to dryness under reduced pressure and the residue (1.29 g) recrystallized from EtOH–H_2O to give an analytical sample: mp 265° dec; ir and nmr as expected; mass spectrum *m/e* 154 (M^+ of the free base). *Anal.* ($C_7H_{10}N_2O_2 \cdot HCl$) C, H, N, Cl.

Enzyme Inhibition Studies. Materials and Methods. Reagent grade chemicals and double-distilled H_2O were used in all enzyme assays. L-Tyrosine-*carboxyl*-^{14}C, L-tryptophan-*carboxyl*-^{14}C, and DL-3,4-dihydroxyphenylalanine-*carboxyl*-^{14}C were purchased from New England Nuclear Corp. L-Tyrosine, dithiothreitol (DTT), and 6,7-dimethyl-5,6,7,8-tetrahydropterine hydrochloride ($DMPH_4$ used in TH assays) were obtained from Calbiochem. L-Phenylalanine was obtained from Sigma Chemical Co. and 2-amino-6,7-dimethyl-4-hydroxy-5,6,7,8-tetrahydropteridine hydrochloride ($AHDMPH_4$ used in PH assays) was obtained from Aldrich Chemical Co.

Phenylalanine Hydroxylase Assay. Partially purified rat liver PH was prepared according to the procedure of Mitoma[19] from female Sprague–Dawley rats, and PH activity was assayed by the method of Bublitz.[14] The standard reaction mixture contained 94 nmol of potassium phosphate (pH 7.4), 12.5 μmol of DTT, 1.25 μmol of $AHDMPH_4$, 1.25 μmol of L-phenylalanine, and inhibitor in 0.2 ml of buffer. The final volume was made to 1.25 ml with H_2O. The reaction was initiated by the addition of a rate-limiting amount of the partially purified enzyme preparation. The reaction mixture was incubated at 37° with shaking for 20 min. The work-up and measurement of tyrosine were performed as previously described in the literature.[20]

Tyrosine Hydroxylase Assay. TH activity was measured using a modification of the coupled decarboxylase assay previously described.[21] TH was prepared from fresh bovine adrenals by the method of Nagatsu, *et al.*,[12] and aromatic L-amino acid decarboxylase was prepared from fresh hog kidneys.[21] The standard reaction mixture contained 100 μmol of sodium phosphate (pH 6.0), 5 nmol of pyridoxal phosphate, 1.2 μmol of $DMPH_4$, 20 μmol of 2-mercaptoethanol, 1.0 μmol of sodium phosphate (pH 7.4), 0.05 μmol of L-tyrosine-*carboxyl*-^{14}C (10 μCi/μmol, 1.1×10^6 dpm), 7.5 units of aromatic L-amino acid decarboxylase (an excess), and 0.23 mg of the TH preparation. The $DMPH_4$ and 2-mercaptoethanol in pH 7.4 sodium phosphate were made fresh daily. Inhibitors were added in 0.10 ml of H_2O and the final volume was made to 0.5 ml with H_2O. The reaction mixture was incubated at 37° with shaking for 20 min. At the completion of the reaction the $^{14}CO_2$ was collected and counted by liquid scintillation spectrometry.[21]

Aromatic L-amino acid decarboxylase was prepared and assayed as described in the literature.[21]

Tryptophan Hydroxylase Assay. Tryptophan hydroxylase activity was measured by the assay procedure of Ichiyama[22] employing a crude mitochondrial preparation from rat brainstem. The standard reaction mixture contained 50 μmol of tris acetate (pH 8.1), 5 nmol of L-tryptophan-*carboxyl*-^{14}C (1.2×10^5 dpm), 77 μmol of sucrose, and 0.15 ml of enzyme. Inhibitors were added in 0.10 ml of H_2O and the final volume was made 0.5 ml with H_2O. The mixture was incubated for 60 min at 37° with constant shaking. The $^{14}CO_2$ was collected and measured as in the TH determination.

Polarography. Polarographic determinations of Fe^{2+} concentrations were made in 0.10 *N* KCl, with a half-wave potential of −1.35 V *vs.* the standard calomel electrode. All determinations were run at pH 7.0 in order to prevent interference with the Fe^{2+} wave.

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