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A NEW CLASS OF TYROSINE HYDROXYLASE INHIBITORS AND A SIMPLE ASSAY OF INHIBITION *IN VIVO**

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Abstract—Tyrosine hydroxylase was shown to be inhibited *in vitro* by a series of phenylcarbonyl derivatives containing catechol or triphenolic ring systems. The most active compounds were either phenethylamine or gallic acid derivatives. A rapid and simple method is described for measuring tyrosine hydroxylase inhibition in the press juice from guinea pig heart after the administration *in vivo* of various compounds. The results obtained with this procedure are consistent with the inhibition of the enzyme *in vivo* as estimated by other procedures.

PREVIOUS reports from this laboratory[1–3] described two classes of inhibitors of tyrosine hydroxylase: (a) α-methyltyrosine and other aromatic amino acid analogues which compete with tyrosine,[1, 2] and (b) catecholamines and 3,4-dihydroxyphenylpropylacetamide (H 22/54) which compete with the pteridine cofactor.[2] α-Methyltyrosine has been shown to be an effective inhibitor *in vivo* of tyrosine hydroxylase in animals[4] and man.[5] Although catechol derivatives are generally less effective inhibitors than tyrosine analogues, H 22/54 is comparable to α-methyltyrosine *in vitro*.[2]

A new class of tyrosine hydroxylase inhibitors, phenylcarbonyl derivatives with catechol or triphenolic ring systems, has been examined and several have been found to be extremely potent *in vitro*. Structure–activity relationships of the more active compounds are described, and a procedure has been developed to evaluate these and other inhibitors after administration to animals *in vivo*.

EXPERIMENTAL

We wish to thank Merck Sharp & Dohme for the α-methyltyrosine and dimethylacetamide, and Winthrop Stearns & Co. for the adrenalone, arterenone, and *N*,*N*-dibenzylarterenone. We also wish to thank Dr. Siro Senoh of Osaka City University, Osaka, Japan, for *N*-acetylarterenone; Drs. J. Axelrod and B. Witkop of the National Institutes of Health, Bethesda, Md., for their gift of *N*-carbobenzoxyarterenone; and Hassle Laboratories, Sweden, for 3,4-dihydroxyphenylpropylacetamide (H 22/54), 3,4-dihydroxyphenylbutylacetamide (H 22/98), and 3,4-dihydroxyphenylethylacetamide (H 22/41). The benzophenone derivatives, benzoic acid derivatives, and pteridine

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cofactor were purchased from the Aldrich Chemical Co. n-Propyl gallate was obtained from Sigma Chemical Co. L-Tyrosine-3,5-^{3}H (5600 μC/μmole) was obtained from New England Nuclear Corp. and purified by passage over Dowex-50. Tyrosine hydroxylase was prepared from beef adrenal medulla according to the procedure of Nagatsu *et al.*[1]

Assay in vitro

Tyrosine hydroxylase activity was assayed by measuring the formation of tritiated water from 3,5-ditritiotyrosine, as described by Nagatsu *et al.*[6] The standard incubation mixture contained 0·5 to 1·0 mg protein, 0·05 μmole tyrosine with 2×10^5 cpm tyrosine-3,5-^{3}H, 0·5 μmole 2-amino-4-hydroxy-6,7-dimethyltetrahydropteridine ($DMPH_4$), 100 μmole mercaptoethanol, 100 μmole phosphate buffer (pH 6·0), and inhibitor in a final volume of 0·5 ml. Inhibitors were dissolved in appropriate solvents and tested at final concentrations of 1×10^{-3} M or less. Incubations were for 15 min at 37° in a metabolic shaker. The reaction was stopped by the addition of 0·1 ml of 25% trichloroacetic acid and the mixture centrifuged. The supernatant solution was placed on a Dowex-50 H^+ column[7] and washed with 1·5 ml water. The effluent and washings were collected in a counting vial and 10 ml Bray's solution[8] added. Radioactivity was determined in a model 4000 Packard TriCarb scintillation counter.

Assay in vivo

Tyrosine hydroxylase inhibition *in vivo* has been measured by analysis of the catecholamine content of tissues[4] or by the conversion of ^{14}C-tyrosine to ^{14}C-norepinephrine.[9] However, assay of inhibition of the catechol derivatives *in vivo* by measurement of the catecholamine content of tissues is difficult because these compounds may either interfere with the chemical determination of catecholamines or release norepinephrine from tissue stores.[10] The isotopic method is expensive and time consuming for screening large numbers of compounds. A simple method has been developed for estimating the inhibition *in vivo* of tyrosine hydroxylase in guinea pig heart by a combination of *in vitro* and *in vivo* techniques.

Appropriate doses of the compounds were dissolved in saline or dimethylacetamide and injected i.p. into Hartley guinea pigs (200–400 g). Guinea pig heart was used because of its high tyrosine hydroxylase activity. After suitable intervals the animals were decapitated and the hearts removed, dissected free from adhering connective tissue, blotted to remove traces of blood, and placed in a chilled stainless steel tissue press (designed by Hogeboom *et al.*[11] and fabricated at the NIH Instrument Shop). Heart muscle was extruded through the 0·5-mm holes in the bottom plate of this press into a chilled polyethylene centrifuge tube. The extruded material (without dilution) was centrifuged at 15,000 *g* for 20 min at 0° to separate the tissue juice from the muscle debris. After this procedure, most of the tyrosine hydroxylase activity was found in the supernatant fluid.[12] An aliquot (usually 0·1 ml) of this fluid was taken for assay of tyrosine hydroxylase activity. An incubation mixture was added containing carrier-free 3,5-^{3}H-tyrosine ($2{\cdot}5 \times 10^5$ cpm), 0·1 μmole $DMPH_4$, 40 μmole mercaptoethanol, and 40 μmole phosphate buffer (pH 6·0) in a final volume of 0·025 to 0·050 ml. Thus, the dilution of substrate and inhibitor in the press juice by the added incubation mixture was never more than 30%. The tyrosine content of guinea pig heart is approximately 15 μg/g. The measured concentration of tyrosine after dilution of press juice with the above incubation mixture ranged from 5×10^{-5} M to 10^{-4} M

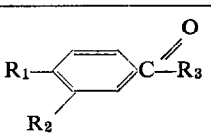
depending on the volumes which were used. The mixture (0·125–0·15 ml, total volume) was incubated for 15 min at 37° in a metabolic shaker and the reaction stopped by the addition of 0·4 ml of 5% trichloroacetic acid. After centrifugation the tritium water which had been formed was assayed as described above. Heated tissue and 0·32 M sucrose solutions were incubated along with the tissue samples in addition to a nonincubated trichloroacetic acid blank. In a typical experiment the incubated blanks gave 350 cpm, the trichloroacetic acid blank 280 cpm, and heart extracts about 7500 cpm.

RESULTS

Studies in vitro

I. Structure–activity relationships of phenylcarbonyl derivatives. A series of phenylcarbonyl derivatives was tested for inhibitory activity against purified tyrosine hydroxylase. The data in Table 1 show that a catechol ring system is required for

TABLE 1. THE EFFECT OF A SERIES OF PHENYLCARBONYL DERIVATIVES ON THE ACTIVITY OF PURIFIED TYROSINE HYDROXYLASE



R_1	R_2	R_3	Concentration	%I
H	H	CH_3	2×10^{-4}	0
H	OH	CH_3	2×10^{-4}	0
HO	H	CH_3	2×10^{-4}	0
H	OH	CH_2CH_3	2×10^{-4}	0
NH_2	H	CH_3	5×10^{-4}	0
H	NH_2	CH_2CH_3	5×10^{-4}	0
CH_3O	HO	CH_3	5×10^{-4}	0
HO	CH_3O	CH_3	5×10^{-4}	0
HO	HO	H	5×10^{-4}	85
HO	HO	CH_3	5×10^{-4}	75
HO	HO	OH	2×10^{-4}	35
HO	HO		5×10^{-4}	95
HO	HO	CH_2NH_2l	2×10^{-5}	35
HO	HO	CH_2NHCH_3[2]	5×10^{-5}	70
HO	HO	CH_2N-Acetyl	2×10^{-4}	45
HO	HO	CH_2N-DBZ[3]	2×10^{-5}	70
HO	HO	CH_2N-CBZ[4]	2×10^{-5}	40

Compounds were added to the standard incubation mixture and incubated at 37° for 15 min as described.
[1] Arterenone.
[2] Adrenalone.
[3] *N,N*-Dibenzylarterenone.
[4] *N*-Carbobenzoxyarterenone.

inhibitory activity. Methylation or removal of either of the phenolic groups abolished all inhibitory activity. Catechol compounds were more potent inhibitors when they contained a β-carbonyl substituent—for example the 3,4-dihydroxy derivatives of benzoic acid, benzaldehyde, and benzophenone. Variations in the side chain

containing the β-carbonyl substituent also influenced inhibitory potency. The most active compounds of this series contained an amine group on the side chain. Acylation of the amine function to form the neutral amide (*N*-acetylarterenone and *N*-carbobenzoxyarterenone) decreased the inhibitory potency. Bulky substituents on the nitrogen increased inhibitory potency (arterenone vs. *N,N*-dibenzylarterenone). The substitution of a hydroxyl, methyl, or hydrogen for the carbonyl function reduced inhibitory activity. Thus adrenalone and noradrenalone, the β-keto analogues of epinephrine and norepinephrine, were far more potent tyrosine hydroxylase inhibitors than the parent compounds.

Studies with diphenolic benzoic acid derivatives demonstrated that hydroxyl groups in the 3 and 4 positions are required for inhibition (Table 2). Compounds with the

TABLE 2. THE EFFECT OF DIPHENOLIC BENZOIC ACID DERIVATIVES ON TYROSINE HYDROXYLASE ACTIVITY *in vitro*

Compound	% Control
2,3-Dihydroxybenzoic acid	105
3,5-Dihydroxybenzoic acid	112
3,4-Dihydroxybenzoic acid	50

The compounds were added to the standard incubation mixture at a concentration of 1×10^{-3} M. The mixture was incubated at 37° for 15 min.

phenolic groups in the 2 and 3 or 3 and 5 positions were devoid of inhibitory activity at the concentrations employed.

A series of triphenolic derivatives was also tested. Table 3 illustrates that this class of compounds has structural requirements for inhibitory potency similar to

TABLE 3. THE EFFECT OF A SERIES OF TRIPHENOLIC COMPOUNDS ON TYROSINE HYDROXYLASE ACTIVITY *in vitro*

Compound added	Final concentration (M)	Dopa formed (mμmoles)	Inhibition (%)
None		16.0	
5-Hydroxydopamine	1×10^{-3}	5·5	66
	5×10^{-4}	9·8	33
	2×10^{-4}	11·8	26
2, 3, 4-Trihydroxyacetophenone	1×10^{-3}	2·0	88
	5×10^{-4}	3·4	80
	2×10^{-4}	6·3	61
5-Hydroxyarterenone	5×10^{-4}	2·3	85
	2×10^{-4}	4·1	75
	1×10^{-4}	6·0	63
n-Propyl gallate	1×10^{-4}	1·3	92
	1×10^{-5}	2·2	86
	2×10^{-6}	7·3	56

The compounds were added to the standard tyrosine hydroxylase incubation mixture and incubated 15 min at 37°.

those of the catechol derivatives. A carbonyl group in the β-position of the side chain increased activity (5-hydroxydopamine vs. 5-hydroxyarterenone). An amine group on the side chain further increased activity (5-hydroxyarterenone vs. 2,3,4-trihydroxyacetophenone). However, the most active compound of this series thus far examined was the *n*-propyl ester of gallic acid. The possibility that *n*-propyl gallate inhibited tyrosine hydroxylase because it is an iron-complexing agent was investigated. However, the addition of a 10-fold excess of Fe^{2+} resulted in only a slight decrease in enzyme inhibition.

II. The mechanism of the effect on purified tyrosine hydroxylase. Fig. 1 shows double-reciprocal plots of $DMPH_4$ concentration vs. the rate of tyrosine hydroxylation, with and without a series of catechol derivatives. It can be seen that catechols

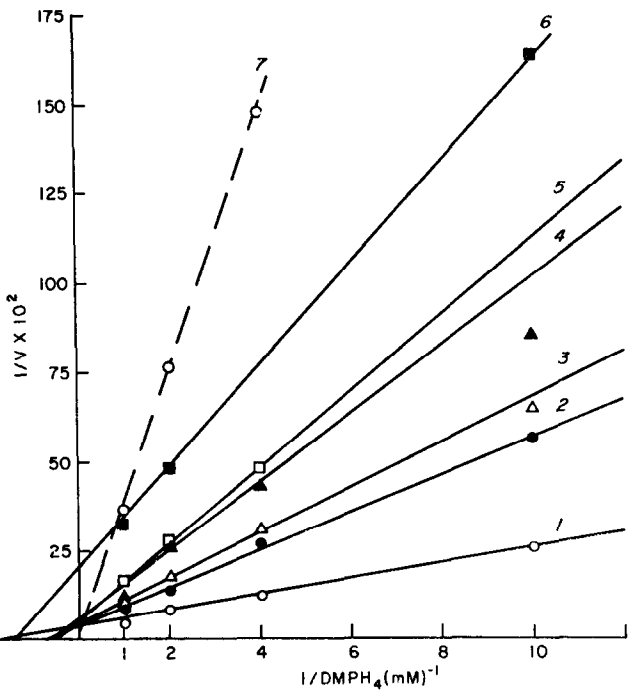


FIG. 1. Double-reciprocal plot of $DMPH_4$ concentration vs. rate of hydroxylation with and without a series of catechols and α-methyltyrosine. The compounds used were: (1) L-tyrosine alone; and L-tyrosine plus (2) arterenone $2{\cdot}5 \times 10^{-5}$ M; (3) norepinephrine 1×10^{-3} M; (4) dopamine 5×10^{-4} M; (5) 3,4-dihydroxybenzoic acid 1×10^{-3} M; (6) α-methyltyrosine 5×10^{-5} M; (7) *N,N*-dibenzylarterenone $2{\cdot}5 \times 10^{-5}$ M. Activity was measured at a tyrosine concentration of 1×10^{-4} M, and $DMPH_4$ varied between 1×10^{-3} M and 1×10^{-4} M.

all exhibited competitive-type inhibition toward $DMPH_4$ whereas α-methyltyrosine, included for comparison, exhibited uncompetitive inhibition.

In a similar study, double-reciprocal plots of the velocity of tyrosine hydroxylation vs. tyrosine concentration with a similar series of inhibitors revealed that catechol derivatives exhibited uncompetitive inhibition toward tyrosine, whereas α-methyltyrosine was a competitive inhibitor. These data are consistent with the results

reported by Udenfriend *et al.*[2] and with the mechanism for tyrosine hydroxylase activity proposed by Ikeda *et al.*[3]

Studies in vivo

I. The validity of the method for estimating inhibition in vivo. Tyrosine hydroxylase inhibitors were assayed for activity *in vivo* by administration to intact guinea pigs and assaying the tyrosine hydroxylase activity in the press juice from the isolated heart. The validity of this approach was first demonstrated with α-methyltyrosine. Table 4 compares tyrosine hydroxylase inhibition estimated in three ways: by the

TABLE 4. COMPARISON OF MEASURED AND CALCULATED TYROSINE HYDROXYLASE INHIBITION IN GUINEA PIG HEART AFTER THE ADMINISTRATION OF α-METHYLTYROSINE

Time (hr)	Measured tyrosine* hydroxylase inhibition (%)	Measured inhibition† of NE synthesis (%)	Calculated tyrosine† hydroxylase inhibition (%)
2	88	92	95
4	87	93	92
8	86	91	96
16		74	87
24	46	56	61
36	13		6

In all experiments 100 mg α-methyltyrosine/kg was administered i.p.

* Tyrosine hydroxylase inhibition was measured *in vitro* after the administration *in vivo* of α-methyltyrosine as described under Experimental.

† Data from S. Udenfriend, *Pharmac. Rev.***18**, 43 (1966).

decreased incorporation of ^{14}C-tyrosine into ^{14}C-norepinephrine; as calculated from the measured concentrations of α-methyltyrosine, tyrosine, and their K_i and K_m values;[13] and by the direct determination of enzyme inhibition with the method described here. The results obtained with all three methods were essentially the same. The slightly lower inhibition observed with the present method is probably due to the small dilution of the tissue juice.

Validity of the method with respect to cofactor antagonists was also checked. The effects produced by several of these inhibitors when added to heart press juice *in vitro* are shown in Table 5.

II. The Effects in vivo of catechol and pyrogallol derivatives. The intraperitoneal administration of the catechol and pyrogallol derivatives studied had little effect on tyrosine hydroxylase activity of guinea pig heart, as shown in Table 6. Large doses of 3,4-dihydroxyphenylacetamide derivatives (H 22/54 and H 22/91), which were previously reported to be active tyrosine hydroxylase inhibitors *in vitro*[2] and *in vivo*[14] were active in this system. However, the carbonyl compounds (*n*-propyl gallate, 3,4-dihydroxybenzophenone, and *N,N*-dibenzylarterenone) which are highly active *in vitro*, failed to inhibit tyrosine hydroxylase when administered to the intact animal. Large doses of dihydroxybenzoic acid derivatives (1000 mg/kg), which were lethal to several animals in the group, were active *in vivo*. This may, however, be a non-specific toxic effect, since 2,3-dihydroxybenzoic acid, which is not an active inhibitor *in vitro*, gave the same results as the 3,4-dihydroxybenzoic acid.

DISCUSSION

Tyrosine hydroxylation is rate limiting[15] in the synthesis of catecholamines from tyrosine, and effective inhibitors for this enzymatic step are valuable pharmacological and biochemical tools. α-Methyltyrosine, an inhibitor that is competitive with the substrate tyrosine, has been used to demonstrate the effect of stress and exercise on turnover times of catecholamines[16] and to lower norepinephrine production in

TABLE 5. THE EFFECT OF VARIOUS COMPOUNDS ON THE TYROSINE HYDROXYLASE ACTIVITY OF PRESS JUICE FROM GUINEA PIG HEART

Addition	Concentration (M)	Inhibition (%)
Phenylalanine	5×10^{-4}	78
α-Methyltyrosine	1×10^{-4}	85
Dopa	5×10^{-4}	47
Dopamine	5×10^{-4}	50
n-Propyl gallate	5×10^{-6}	46
N, N-Dibenzylarterenone	5×10^{-5}	51

Press juice from guinea pig heart (0·1 ml) was preincubated with the inhibitor for 5 min at 37°. Then 0·05 ml of the standard *in vivo* incubation mixture was added and incubation was continued for an additional 15 min. Activity was measured as described under Experimental.

TABLE 6. TYROSINE HYDROXYLASE ACTIVITY IN PRESS JUICE FROM GUINEA PIG HEART AFTER ADMINISTRATION OF VARIOUS CATECHOLS

Compound	Dose (mg/kg)	cpm isolated per heart	Inhibition (%)
H 22/54	625	2867	65
H 22/98	625	7138*	5
H 22/41	750	1550	83
3,4-Dihydroxybutyrophenone	250†	7106	6
3,4-Dihydroxybenzoic acid	1000	3565	54
2,3-Dihydroxybenzoic acid	1000	3776	52
N,N-Dibenzylarterenone	1000	7480	0
n-Propyl gallate	200†	8187	0
n-Propyl gallate	400†	8319	0
None		7617	
Dimethylacetamide (solvent)		7425	0

Three guinea pigs (300 g) were injected i.p. with the various catechols dissolved in 0·8 ml dimethylacetamide. The animals were sacrificed after 90 min and the tyrosine hydroxylase activity in the heart press juice assayed as described under Experimental.

* One animal used.

† Animals treated with 500 and 1000 mg/kg died shortly after injection.

patients with pheochromocytoma and hypertension.[5] The inhibitors described in this report are competitive with cofactor and require for maximal activity phenolic groups in the 3 and 4 positions and a carbonyl group on the β-carbon. An amine group in the side chain increases inhibitory activity but is not required for inhibition since derivatives of benzoic acid, benzaldehyde, and acetophenone are potent inhibitors. Studies with triphenolic derivatives have shown that the structure–activity relationship of this class of compounds is similar to that of the catechols. The most

potent inhibitor of the entire group studied thus far, *n*-propyl gallate, is of additional interest because of its use as a food additive approved for human consumption.[17]

The reason for the marked increase in tyrosine hydroxylase inhibition found on substitution of a β-carbonyl group for a β-hydroxyl group or a β-hydrogen atom is not apparent. However, it may be due to electronic changes in the catechol ring system. The electronic interaction of the β-carbonyl with the phenolic groups significantly decreases the pK_a of the *p*-hydroxyl group. The increased acidity of this phenolic group may increase the strength of binding to the enzyme.

The failure to observe significant inhibition of tyrosine hydroxylase on administration of these compounds *in vivo* was disappointing and indicates that adequate tissue levels were not attained even though enormous doses were administered. In some cases, enough must have been absorbed to produce lethal effects. It appears that the polyphenolic compounds which were used in these studies are rapidly metabolized and/or excreted. Conceivably, there may also have been poor absorption from the site of injection. Preliminary results with an ultraviolet absorption method for assay of *N,N*-dibenzylarterenone in tissues corroborate these conclusions.

The potent effect *in vitro* of these carbonyl derivatives is of great interest despite the lack of tyrosine hydroxylase inhibition in *vivo*. It is possible that modifications of their structure may alter absorption and metabolism and result in compounds which are active *in vivo*.

The technique of administering a compound to an intact animal and assaying the appropriate enzyme *in vitro* permits the rapid estimation of their effectiveness *in vivo*. The method described here minimizes dilution of substrate and inhibitor, which is inherent in homogenization procedures, by using undiluted heart press juice from guinea pig and tracer amounts of radioactive tyrosine. Thus, tyrosine hydroxylase activity can be measured at approximately the substrate and inhibitor concentrations that exist *in vivo*. The carbonyl derivatives could not be assayed under exact *in vivo* conditions because the guinea pig heart press juice requires fortification with $DMPH_4$ and the status of the cofactor in the heart is not known.

The validity of the *in vivo* method was shown by the rapid and marked enzyme inhibition observed after the administration of α-methyltyrosine. Similarly, inhibition of tyrosine hydroxylase was observed after the administration *in vivo* of the phenylacetamides (H 22/54 and H 22/41). These compounds compete with cofactor[2] and decrease tissue norepinephrine levels *in vivo*.[13, 14]

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