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# TYROSINE HYDROXYLASE INHIBITION *IN VITRO* AND *IN VIVO* BY DEOXYFRENOLICIN

R. J. TAYLOR, JR.,* C. S. STUBBS, JR. and LEON ELLENBOGEN

Lederle Laboratories, American Cyanamid Company, Pearl River, N. Y. 10965, U.S.A.

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**Abstract**—Deoxyfrenolicin, an analog of frenolicin, an antibiotic produced by *Streptomyces fradiae*, was shown to be a potent inhibitor of tyrosine hydroxylase. Kinetic studies indicated that inhibition was competitive with tyrosine, was increased by high concentrations of pteridine cofactor, and could not be reversed by $Fe^{2+}$. When administered to rats at 50 mg/kg, deoxyfrenolicin significantly inhibited adrenal tyrosine hydroxylase activity. The naphthoquinone structure of deoxyfrenolicin represents a novel structure with tyrosine hydroxylase-inhibiting properties.

TYROSINE hydroxylase, the enzyme which catalyzes the initial step in the biosynthesis of norepinephrine, has been found to occur in brain, adrenal medulla and other sympathetically innervated tissues[1, 2] and to require a specific pteridine cofactor.[3, 4] A variety of types of compounds have been reported to inhibit tyrosine hydroxylase *in vitro*. Certain aromatic amino acids inhibit the enzyme by competing with the substrate tyrosine.[5] A large number of catechols,[5–7] the catechol isostere, 4-isopropyltropolone,[8] and certain indole amino acids[9] inhibit by competing with the pteridine cofactor. A series of metal chelating agents[10] and pyrroloisoxazoles[11] appear to inhibit by complexing with a required metal ion. The present communication describes the inhibition of tyrosine hydroxylase *in vitro* and *in vivo* by deoxyfrenolicin. Deoxyfrenolicin (Fig. 1) is an analog of frenolicin, an antibiotic produced by *Streptomyces fradiae*, and has a naphthoquinone structure.[12, 13]

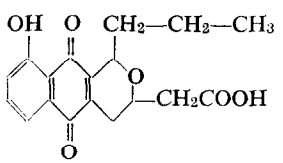


FIG. 1. Structure of deoxyfrenolicin.

## EXPERIMENTAL

### *Materials*

The deoxyfrenolicin used in this study was supplied by Drs. M. Kunstmann and G. A. Ellestad of these laboratories and was in crystalline form (99+ % pure). α-Methyl-*p*-tyrosine was obtained from Merck, Sharp & Dohme Research Laboratory; 3,4-dihydroxyphenylpropylacetamide (H-22/54) from Hassle Laboratories, Sweden;

* Present address: Department of Biochemistry, McNeil Laboratories, Fort Washington, Pa. 19034.

and 2-2′-bipyridyl from G. Frederick Smith Chemical Company. Freshly prepared aqueous solutions of ferrous ammonium sulfate were used as the source of $Fe^{2+}$.

*Assay* in vitro

Tyrosine hydroxylase was prepared from bovine adrenal medulla by the procedure of Nagatsu *et al.*[1] The enzyme was precipitated from the 105,000 *g* supernatant by addition of ammonium sulfate to 40 per cent saturation, and the precipitate was resuspended in $1 \times 10^{-3}$ M aqueous mercaptoethanol (pH 6·5) and dialyzed overnight against 200 vol. of deionized water at 5°. The dialyzed preparation contained about 30 mg protein per ml. Tyrosine hydroxylase activity *in vitro* was assayed by the procedure developed by Nagatsu *et al.*[14] The standard incubation mixture consisted of 0·1 μmole L-tyrosine containing $2 \times 10^4$ cpm L-tyrosine-3,5-$^3$H (New England Nuclear), 200 μmoles acetate buffer (pH 6·0), 1·0 μmole 2-amino-4-hydroxy-6,7-dimethyltetrahydropteridine ($DMPH_4$) in 0·1 M phosphate (pH 7·4) containing 1 M mercaptoethanol, 0·1 μmole 4-bromo-3-hydroxybenzyloxamine dihydrogen phosphate (NSD-1055), and 0·2 ml of enzyme preparation. The deoxyfrenolicin was added to the reaction mixture in 0·1 ml of 50% aqueous dimethylsulfoxide. The final volume was made to 1·0 ml with water. All incubations were for 30 min at 37° in a metabolic shaker. The reaction was stopped by the addition of 0·05 ml of glacial acetic acid and the mixture was centrifuged. The supernatant solutions were placed on Dowex-50($H^+$) columns[4] and washed with 1·0 ml water. The effluents and washings were mixed with 10 ml Bray's solution, and radioactivity was determined in a Packard Tri-Carb liquid scintillation counter. Controls were included to correct for nonenzymatic hydroxylation and for inhibition due to the solvent.

*Assay* in vivo

Tyrosine hydroxylase activity was estimated in rat adrenals, as described in an earlier report.[11] The deoxyfrenolicin was administered intraperitoneally to male Sprague–Dawley rats (Carworth Farms, 100–120 g) which had been fasted for 18 hr.

The animals were decapitated after 3 hr and the adrenals removed, freed of connective tissue and weighed. A 20% (w/v) homogenate of the pair of adrenals from each rat was prepared in 0·32 M sucrose and centrifuged at 10,000 *g* for 20 min at 0°. Tyrosine hydroxylase activity was measured in a 0·02-ml aliquot of the supernatant. The remaining incubation mixture contained 40 μmoles of phosphate buffer (pH 6·0), 40 μmoles mercaptoethanol, 0·1 μmole $DMPH_4$ and carrier-free L-tyrosine-3,5-$^3$H ($1{\cdot}25 \times 10^{-4}$ μmoles, $2{\cdot}2 \times 10^4$ cpm) in a volume of 0·05 ml. The total mixture of 0·07 ml was incubated for 20 min at 37° in a metabolic shaker, and the reaction was stopped by the addition of 0·4 ml of 5% (w/v) trichloroacetic acid. After centrifugation, the tritiated water formed was assayed *in vitro* by the procedure described above.

## RESULTS

A comparison of the inhibition of tyrosine hydroxylase *in vitro* by deoxyfrenolicin and other tyrosine hydroxylase inhibitors is shown in Table 1. The molar $I_{50}$ values were obtained from a plot of the points obtained from the mean of duplicate analyses of at least three inhibitor concentrations. Deoxyfrenolicin is a potent inhibitor of the bovine adrenal enzyme *in vitro*, having an $I_{50}$ of $8 \times 10^{-5}$ M. The compound is

slightly more potent than CL 65,263 and H-22/54, but less potent than 2,2′-bipyridyl, α-methyl-*p*-tyrosine and 3-iodo-L-tyrosine.

Double reciprocal plots of tyrosine concentration versus the rate of tyrosine hydroxylation indicated that the inhibition by deoxyfrenolicin was competitive with tyrosine at tyrosine concentrations from $4 \times 10^{-6}$ to $1 \times 10^{-4}$ M (Fig. 2). Deoxyfrenolicin also appears to be uncompetitive with $DMPH_4$ over a range of $DMPH_4$ concentrations from $5 \times 10^{-4}$ to $1 \times 10^{-3}$ M (Fig. 2). However, at concentrations of greater than $1 \times 10^{-3}$ M, a deviation from linearity is observed, indicating greater inhibition by the deoxyfrenolicin. A similar observation with $DMPH_4$ was reported with the inhibitor aquayamycin.[15]

As shown in Table 2, addition of 0·5 μmole $Fe^{2+}$ to the incubation mixture failed to reverse the inhibition due to $5 \times 10^{-5}$ M or $1 \times 10^{-4}$ M deoxyfrenolicin. Addition of $Fe^{2+}$ has been reported[10, 11] to reverse the inhibition due to *o*-phenanthroline and CL 65,263, but not H-22/54 or α-methyl-*p*-tyrosine.

Intraperitoneal administration of deoxyfrenolicin to rats resulted in a 36 per cent reduction of adrenal tyrosine hydroxylase activity in 3 hr at a dose of 50 mg/kg (Table 3). No significant inhibition was observed at 10 or 25 mg/kg.

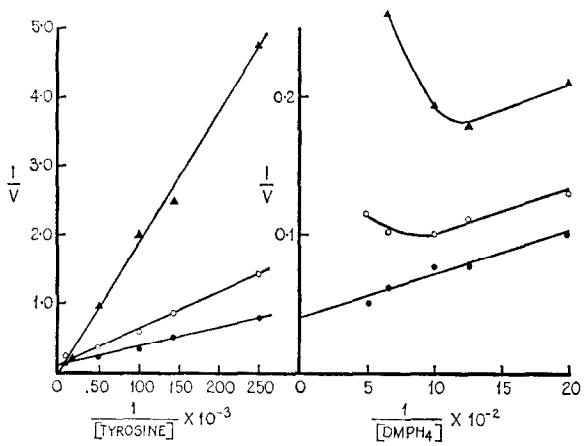


FIG. 2. Double reciprocal plots of tyrosine concentration or $DMPH_4$ concentration versus rate of tyrosine hydroxylation with no deoxyfrenolicin (●—●) and with deoxyfrenolicin at $5 \times 10^{-5}$ M ○—○) and $1 \times 10^{-4}$ M (▲—▲). At varied tyrosine concentrations, $DMPH_4$ was $1 \times 10^{-3}$ M; at varied $DMPH_4$ concentrations, tyrosine was $1 \times 10^{-4}$ M.

TABLE 1. COMPARISON OF DEOXYFRENOLICIN WITH OTHER TYROSINE HYDROXYLASE INHIBITORS

| Compound | Molar $I_{50}$ |
|---|---|
| Deoxyfrenolicin | $8 \times 10^{-5}$ |
| 3-Amino-4H-pyrrolo[3,4c]isoxazole-5(6H)-carboxylic acid, ethyl ester (CL 65,263) | $1 \times 10^{-4}$ |
| 3,4-Dihydroxyphenylpropylacetamide (H-22/54) | $1 \times 10^{-4}$ |
| 2,2′-Bipyridyl | $1 \times 10^{-5}$ |
| DL-α-Methyl-*p*-tyrosine | $6{\cdot}5 \times 10^{-5}$ |
| 3-Iodo-L-tyrosine | $2 \times 10^{-6}$ |

TABLE 2. EFFECT OF $Fe^{2+}$ ON THE INHIBITION OF TYROSINE HYDROXYLASE BY DEOXYFRENOLICIN

| | $Fe^{2+}$ added | | | |
|---|---|---|---|---|
| | 0 | | 0·5 μmole | |
| Inhibitor | mμmoles Dopa* formed | % Inhibition | mμmoles Dopa formed | % Inhibition |
| None | 5·1 | | 7·2 | |
| $5 \times 10^{-5}$ M Deoxyfrenolicin | 3·7 | 27 | 5·3 | 26 |
| $1 \times 10^{-4}$ M Deoxyfrenolicin | 2·4 | 53 | 3·4 | 53 |

* Dopa = 3,4-dihydroxyphenylalanine.

TABLE 3. TYROSINE HYDROXYLASE ACTIVITY IN RAT ADRENALS AFTER ADMINISTRATION OF DEOXYFRENOLICIN

| Dose (mg/kg, i.p.) | Tyrosine hydroxylase activity* | Inhibition (%) |
|---|---|---|
| 0 | 8·84 ± 0·50 | |
| 10 | 7·66 ± 1·85 | 13 |
| 25 | 8·33 ± 0·44 | 6 |
| 50 | 5·67 ± 0·76 | 36 ($P < 0·05$†) |

* Expressed as per cent of $^3$H-tyrosine hydroxylated. Initial concentration of tyrosine was $1·245 \times 10^{-4}$ μmoles/ml. Values represent the average for five rats ± S. E.

† Determined by the Student *t*-test.

## DISCUSSION

The data presented in this report indicate that deoxyfrenolicin is a potent inhibitor of tyrosine hydroxylase both *in vitro* and *in vivo*. *In vitro*, the compound inhibited 50 per cent at $8 \times 10^{-5}$ M and was nearly equal in potency to α-methyl-*p*-tyrosine and 2,2′-bipyridyl, and more potent than H-22/54 and CL 65,263. The kinetic studies indicated that inhibition was competitive with tyrosine and that inhibition could be increased with excess $DMPH_4$. No reversal of inhibition was observed upon adding $Fe^{2+}$. These data suggest a novel mechanism of inhibition for this compound. Since $DMPH_4$ is not the natural cofactor and is present in stoichiometric amounts in the assay *in vitro*, its relationship to the mechanism *in situ* is difficult to predict. The fact that this inhibitor is competitive with tyrosine and is not structurally related to tyrosine adds more to its uniqueness.

*In vivo*, deoxyfrenolicin gave significant inhibition (36 per cent) at doses of 50 mg/kg. This is comparable to the 52 per cent inhibition observed with 50 mg/kg of α-methyl-*p*-tyrosine, as previously reported,[11] and is greater than the inhibition *in vivo* by CL 65,263. The latter compound was previously found to inhibit 45 per cent at 100 mg/kg.[11] These data indicate deoxyfrenolicin to be one of the most potent inhibitors *in vivo* of tyrosine hydroxylase yet reported. The effect of the compound on endogenous catecholamine levels has not been determined. There have been no reports to date on the mechanism of action of deoxyfrenolicin as an antifungal agent or on the effects of the antibiotic on mammalian metabolic pathways. The compound was recently reported to influence adipocyte metabolism.[16, 17]

The naphthoquinone structure of deoxyfrenolicin represents another new chemical structure with tyrosine hydroxylase-inhibiting properties. The antibiotic, aquayamycin, which may also be a quinone,[18] has also been reported to be a potent tyrosine hydroxylase inhibitor *in vitro*.[15]

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