EFFECT OF ANALOGUES ON THE GROWTH AND TRYPTOPHAN SYNTHETASE OF *PSEUDOMONAS AERUGINOSA*[1]

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Abstract: 5-Fluorotryptophan, a strong inhibitor of *Pseudomonas putida* at a concentration of 200 μg/ml, had an inhibitory effect on *Pseudomonas aeruginosa* (PA-1), but even at very high concentration the inhibition was still incomplete. On the other hand, another analogue, 5-fluoroindole, showed complete inhibitory effect on both PA-1 and *P. putida* at a concentration of 50 μg/ml.

Tryptophan, the end product of tryptophan biosynthetic pathway, showed a little feedback inhibition on tryptophan synthetase. And the inhibition was less effective on the analogue-resistant mutants: 5-FIR-12 and 5-FTR-1, than on the wild type. 5-Fluoroindole could be substituted for indole as the substrate of tryptophan synthetase. But 5-fluoroindole gave the enzyme a partially competitive inhibition with respect to serine. Whereas 5-fluorotryptophan showed inhibition on both substrates: indole and serine. The inhibitory effect was found to be proportional to the concentration of indole. And this analogue also exhibited a partially competitive inhibition with respect to serine.

From the results, the conclusion can be drawn that 5-fluoroindole does not inhibit PA-1 mainly by its conversion to 5-fluorotryptophan, but rather it may affect the cells directly or by its conversion to other strong inhibitors.

INTRODUCTION

It has been found by the studies of diverse biosynthetic and degradative pathways that the gene organization and its regulation are varied in different microorganisms. For example, the structural genes of eneymes for the biosynthesis of methionine, isoleucine and valine are linked to each other on the chromosome in *Escherichia coli* (Demerec, M., 1964), and are dispersed on the chromosome in *Peudomonas aeruginosa* (Fargie and Holloway, 1965). However, the structural genes for arginine biosynthesis are scattered in *E. coli* (Gorini and Gunderson, 1961).

With respect to the types of control, end-product repression, end-product induction, and precursor activation (or induction) within different pathways were reported by Horvath, Varga, and Szentirmai (1964), Gorini and Gunderson (1961), and Sanwal (1970).

Because the biosynthetic pathway of tryptophan offers several advantages for a study of the regulation of anabolic enzymes, among them opportunity to introduce the intermediate compounds anthranilate and indole into cells, and ease of detecting as accumulation products anthranilate, 1-(o-carboxyphenylamino)-1-deoxyribulose, indoleglycerol, and indole. This pathway has been worked out in quite detail. In investigation the genes and enzymes of the tryptophan

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pathway of various bacteria and fungi, some remarkable dissimilarities in the organization of the genes on the chromosome and in the regulatory mechanisms have been observed. *Neurospora* and *Pseudomonas* are different from some other groups such as the enteric bacteria. In the former the genes are dispersed (Maurer and Crawford, 1971) and in the latter clustered (Jackson and Yanofsky, 1972). The enzyme of the last step of this pathway, tryptophan synthetase, consists of two protein subunits in the enterics (Yanofsky, C., 1960) and *P. putida* (Gunsalus *et al.*, 1968). A-subunit converts indoleglycerol phosphate into indole and triose phosphate, and B-subunit combines indole and serine to form tryptophan. While in *N. crassa*, there is only one protein unit which catalyzes both reactions (Yanofsky, C., 1960).

The mechanism for the regulation of the pathway is also different in these microorganisms. In the enterics, the synthesis of all enzymes is controlled coordinately by the excessive end-product, tryptophan (Ito and Crawford, 1965). However, in *P. putida*, tryptophan acts as a repressor only on the expression of the first three linked genes and does not affect the others (Maurer and Crawford, 1971). Tryptophan synthetase may be induced by its substrate, indole-glycerol phosphate, as was reported for *P. putida* and *N. crassa* (Crawford and Gunsalus, 1966; Lester, G., 1971).

The activity of tryptophan synthetase in *P. aeruginosa* has been studied previously in our laboratory and several analogues of tryptophan and its biosynthetic intermediates have been used as tools (Tsai and Liu, 1975). In this paper, we chose 5-fluoroindole and 5-fluorotryptophan from eight inhibitory analogues used for the study to test whether these have any effect on the growth and on the tryptophan synthetase activity in *P. aeruginosa.*

MATERIAL AND METHODS

Bacterial Strain: Strain 1 of *Pseudomonas aeruginosa* (designated as PA-1) obtained from the Department of Microbiology, Oklahoma State University, was used for the studies. Analogue-resistant mutants were isolated from parental PA-1 strain after treatment with N-methyl-N′-nitro-nitrosoguanidine (MNNG).

Medium: Vogel and Bonner's (1956) minimal medium supplemented with 0.2% glucose was used. All components were sterilized by millipore filtration. Other special chemicals in the medium were sterilized separately and added to the medium as the experiment required. If solid medium was desired, 1.5% of autoclaved agar was added.

Growth Condition: The cells were washed with the glucose minimal medium from overnight grown agar slant into the same medium and incubated at 37°C in the reciprocal shaker with 128 vibration per minute.

Growth Curve: Cell turbidity was determined by Bauch & Lomb Spectronic 20 at 540 nm.

Resistant Mutants Isolation: 10^9 wild type cells per milliliter grown in glucose minimal medium were mutagenized with MNNG at a final concentration of 100 μg/ml for one hour at 37°C and then spreaded on glucose minimal medium agar plates containing the analogues, 5-fluoroindole (100 μg/ml), and 5-fluorotryptophan (1 mg/ml) respectively. After 2-3 days of incubation, the colonies appearing on the plates were isolated and purified by two sequential streakings on the same kind of agar plate.

Detection of Intermediates Accumulation: Anthranilate accumulation in culture was quantitated by fluorometer (G. K. Turner Associates model No. 110) at a wave length of 405 nm (activated at 300-400 nm) after extraction from the cells (Crawford and Gunsalus, 1966; Smith and Yanofsky, 1962). Indoleglycerol phosphate was indirectly measured by detecting the concentration of indole that was released by heating the indoleglycerol phosphate in culture filtrate or in crude extract with diluted NaOH at 100°C for 15 minutes (Dawson *et al.*, 1969; Turner and Matchett, 1968).

Crude Extract Preparation and Enzyme Assay: Crude extract was prepared as previously described (Tsai and Liu, 1975) except that 10 mM β-mercaptoethanol and 38 μM pyridoxal phosphate was added to 0.1 M potassium phosphate buffer (pH 7.0) and 10 mM β-mercaptoethanol and 0.8 M sucrose was added to 0.1 M potassium phosphate buffer (pH 7.8). The enzyme activity was assayed according to the method of Smith and Yanofsky (1962) in phosphate buffer or in NaCl supplemented Tris buffer. One enzyme unit is defined as the consumption of 0.1 μmole of substrate in 20 minutes at 37°C. Specific activity is expressed as unit of enzyme activity per mg protein. Protein concentration was determined by the method of Lowry *et al.* (1951).

RESULTS

Effect of Analogues on Growth: Among the analogues used only 7-methylindole, 5-methylindole, 5-fluoroindole, and 5-fluorotryptophan exhibited inhibition on the growth of PA-1 (Table 1). Generally speaking, *Pseudomonas* strains often tolerate high levels of metabolic inhibitors that are toxic to enteric bacteria (Maurer and Crawford, 1971). The three indole substitutes therefore, were strong inhibitors. The strongest inhibitor, 5-fluoroindole was chosen for further study. Meanwhile, 5-fluorotryptophan was also used in order to find out the relationship between these two analogues.

Resistant Mutant Isolation and Their Characteristics: After treatment of MNNG, a number of mutants were isolated. Three 5-fluoroindole and two 5-fluorotryptophan resistants (designated as 5-FIR and 5-FTR respectively), which accumulated more anthranilate than any of the other were used for tests.

Table 1 shows that the concentration of 5-methylindole and 7-methylindole required for inhibiting the growth of PA-1 were much higher than that of 5-fluoroindole. In the cross-resistance test, both 5-FIR's and 5-FTR's showed the same resistance to 5-fluoroindole or 5-fluorotryptophan. And to 5-methylindole or 7-methylindole, they showed nearly the same resistant capability as the wild type (Table 2). Consequently, it is very possible that these two kinds of resistant mutants fall into the same category (Maurer and Crawford, 1971).

We conducted the following physiological experiments: indole production, nitrate reduction, citrate and carbohydrate utilization, starch and gelatin hydrolysis, litmus milk reaction, oxidase

Table 1. The effect of various analogues on the growth of *Pseudomonas aeruginosa*

Analogues	Corcentation (μg/ml)	Growth
5-methyl-tryptophan	1000	++++
6-methyl-tryptophan	1000	+++
Indole acrylic acid[a]	1000	++++
Indole propionic acid	1000	++++
7-methyl-indole	300	—
5-methyl-indole	300	—
5-fluoro-indole	100	—
5-fluoro-tryptophan	1000	+

PA-1 cells were prepared as described in the text. Cultures shaken at 37°C for 18 hours were recorded as ++++ normal, +++ medium, ++ slight, + very slight and—no growth.

[a] This analogue was dissolved in 0.2 N NaOH solution. The sterilization was performed by filtration with millipore.

Table 2. Cross-resistance among analogue-resistants of *Pseudomonas aeruginosa*

Strains	none	Analogues (μg/ml) 5-FI 50	75	100	150	200	5-MI 50	75	100	150	200
Wild type	⧻	–	–	–	–	–	⧻	⧻	⧺	±	–
5-FIR-4	⧻	⧺	+	–	–	–	⧺	⧺	+	±	–
5-FIR-10	⧻	⧺	+	–	–	–	⧺	⧺	+	±	–
5-FIR-12	⧻	⧺	+	–	–	–	⧺	⧺	+	±	–
5-FTR-1	⧻	+	+	–	–	–	⧻	⧻	⧺	±	–
5-FTR-2	⧻	+	+	–	–	–	⧻	⧻	⧺	±	–

Strains	none	Analogues (μg/ml) 7-MI 50	75	100	150	200	5-FT 500	600	700	800	900	1000	
Wild type	⧻	⧻	⧻	⧺	±	–	+	+	+	+	+	+	+
5-FIR-4	⧻	⧺	⧺	+	±	–	⧻	⧻	⧻	⧻	⧻	⧻	⧻
5-FIR-10	⧻	⧺	⧺	+	±	–	⧻	⧻	⧻	⧻	⧻	⧻	⧻
5-FIR-12	⧻	⧺	⧺	+	±	–	⧻	⧻	⧻	⧻	⧻	⧻	⧻
5-FTR-1	⧻	⧻	⧻	⧺	±	–	⧻	⧻	⧻	⧻	⧻	⧻	⧻
5-FTR-2	⧻	⧻	⧻	⧺	±	–	⧻	⧻	⧻	⧻	⧻	⧻	⧻

0.05 ml of 1:10 dilution of a stationary-phase culture in glucose mininal medium was used to inoculate 5 ml of the same medium containing the indicated amount of analogues. Cultures were grown for 18 hr. at 37°C with shaking. Growth was recorded as ⧻ normal, ⧺ medium, + slight, ± varied, and-no growth.

and catalase production tests to compare the mutants with wild [type cells. We found the mutants differed from the parental strain only in the loss of the capability to hydrolyze gelatin and that the 5-FIR mutants also lost their chromgenic property. In the experiments of intermediate accumulation, two kinds of mutants accumulated more anthranilate in the cells than did the wild type. And neither of the mutants nor the wild type accumulated indoleglycerol phosphate in culture filtrates or in crude extracts (Table 3).

Table 3. The accumulation of anthranilate & indoleglycerol phosphate by wild type and analogue resistants.

Growth medium	Analogues added (μg/ml)	Strains: Wild type A. A.[a] excreted	InGP[b]	5-FIR-12 A. A.[a] excreted	InGP[b]	5-FTR-1 A. A.[a] excreted	InGP[b]
Glu. m. m.	none	1.8×10^{-9}	0	1.71×10^{-7}	0	4.55×10^{-8}	0
Glu. m. m.	5-FI (50)	—	0	—	0	—	0
Glu. m. m.	5-FT (1000)	—	0	—	0	—	0

Methods were described in the text.

Abbreviations used: A. A. anthranilate, InGP. indoleglycerol phosphate

[a] A. A. was calculated as nmole/ml/cell.

[b] InGP was measured Both inside and outside the cell.

—, Not detect

Figs. 1, 2, 3 show the growth curves for 5-FIR-12, 5-FTR-1 and parental strain when grown in the presence or absence of analogues respectively. 5-Fluorotryptophan (1 mg/ml) is not an inhibitor to 5-FIR-12 and 5-FTR-1, although it inhibits the parental strain to a large extent (Fig. 2). On the other hand, 5-fluoroindole at a relatively low concentration (50 μg/ml) completely inhibits the growth of the wild type, and also shows an inhibitory effect on 5-FTR-1, but not completely. 5-FIR-12, after a longer lag phase, can still grow to the maximum turbidity.

Effect of Tryptophan, 5-Fluorotryptophan, and 5-Fluoroindole on the Synthesis and Activity of Tryptophan Synthetase: Tryptophan, the end-product of the pathway, showed partially feed-back repression on the enzyme (Table 4). 5-Fluoroindole can substitute for indole as a substrate. But 5-fluorotryptophan is an effective inhibitor for the enzyme (Table 5).

Fig. 4 and 5 show that at a constant concentration of serine (3.7×10^{-2} M), 5-fluoroindole shows no effect on inhibition with various concentration of indole. However, in the presence of 5-fluorotryptophan, increasing concentration of indole appears to increase the relative effect of inhibition. On the contrary, as indole concentration holds constant (2.5×10^{-4} M), both 5-fluoroindole and 5-fluorotryptophan exhibit partially competitive inhibition on the enzyme when serine is presented at various concentrations (Fig. 6 and Fig. 7).

DISCUSSION

PA-1 is highly tolerant to analogues of tryptophan and of its biosynthetic intermediates (Table 1). Among eight analogues tested, we found that 5-fluoroindole had the strongest inhibitory effect, while the analogues which were the inhibitors to the growth of other micro-

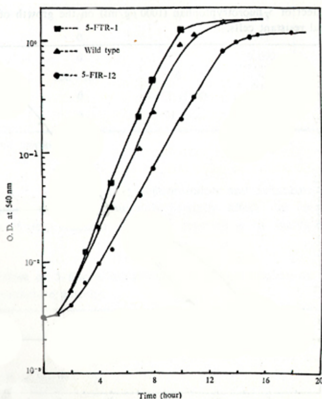


Fig. 1. Growth of wild type and two analogue resistant mutants on glucose minimal medium.

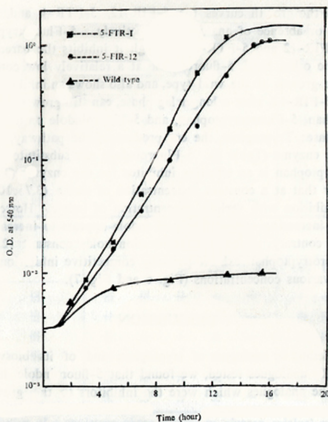


Fig. 2. Effect of 5-fluorotryptophan (1000 μg/ml) on the growth of wild type and resistant cells.

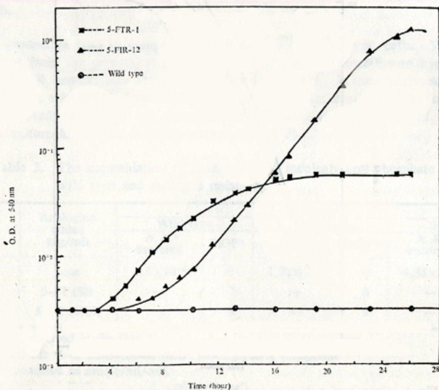


Fig. 3. Effect of 5-fluoroindole (50 μg/ml) on the growth of wild type and resistant cells.

organisms such as 5-methyltryptophan (Moyed, H. S., 1960) and indoleacrylic acid (Matchett, W. H., 1972) had no effect on this strain. It may be that PA-1 possesses a permeability barrier for these analogues, an enzyme that may change these analogues to normal products or the target site may be insensitive to these compounds. It is also interesting that 5-fluorotryptophan strongly inhibits the growth of *P. putida* (Maurer and Crawford, 1971), however, it has a very slight effect on PA-1.

Maurer and Crawford (1971) suggested that the inhibition of 5-fluoroindole on *P. putida* was due to the conversion to 5-fluorotryptophan which was the real growth inhibitor. The inhibitory effect of 5-fluorotryptophan at a concentration of 1 mg/ml is still incomplete on PA-1. However, 5-fluoroindole completely inhibits PA-1 at a much lower concentration (50 μg/ml) (Table 1, Fig. 2 and Fig. 3).

Table 4 shows that the end-product repressed the enzyme slightly. And the addition of tyrosine and phenylalanine did not strengthen this effect. In *E. coli*, the addition of these two amino acids to the medium would suppress the formation of tryptophanase and chorismate dimutase which would interfere with assays of low levels of trp operon-specified enzymes (Morse and Yanofsky, 1969). Since there is no tryptophanase in PA-1, it may be that the chorismate mutase is not affected by these two amino acids in PA-1.

Table 4. Effect of tryptophan, tyrosine, phenylalanine, and 5-fluorotryptophan, 5-fluoroindole on the tryptophan synthetase enzyme level.

Growth medium	Supplement with tryptophan (μg/ml.)	Addition of tyr. and phe. (μg/ml.)	Analogues added (μg/ml.)	Specific activity (units/mg of protein)		
				Wild type	5-FTR-1	5-FIR-12
Gmm.	0	0	0	0.84	0.77	0.74
Gmm.	20	0	0	0.81	0.73	0.72
Gmm.	100	0	0	0.699	0.7	0.7
Gmm.	100	100+100	0	0.694	0.72	0.69
Gmm.	0	0	5-FT (1000)	—	0.75	0.76
Gmm.	0	0	5-FI (50)	—	—	0.73

Gmm: Glucose minimal medium
—: not detect.

Table 5 and Figs. 4, 5, 6, 7 show that 5-fluoroindole and 5-fluorotryptophan do not affect the combination of enzyme and indole, but only partially affect the reaction of enzyme and serine. The inhibition of the reaction could be reversed with increasing the concentration of serine.

Table 5. Effect of 5-fluoro-indole and 5-fluoro-tryptophan on tryptophan synthetase activity

Analogues added to reaction mixture	Specific activity (units/mg of protein)		
	wild type	5-FIR-12	5-FTR-1
5-FI (50 μg/ml) instead of indole pre-existed in reaction mixture	0.7	0.78	0.72
5-FI (50 μg/ml)	0.84	0.76	0.79
5-FT (1000 μg/ml)	0.42	0.44	0.43

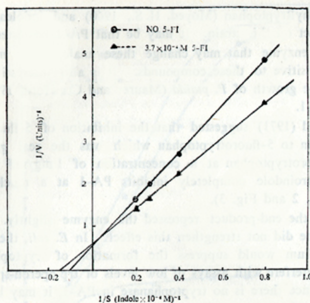
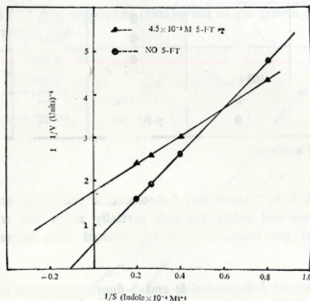


Fig. 4. Kinetics of inhibition by 5-fluoroindole. Indole is the limiting substrate, serine is present at optimal concentration (3.7×10^{-2} M). 5-Fluoroindole was added to a final concentration of 3.7×10^{-4} M. The data were subjected to linear regression analysis (Seteel & Torrie, 1960). Km for the uninhibited reaction=0.91×10^{-3} M; for the presence of inhibitor=0.66×10^{-3} M.



Eig. 5. Kinetics of inhibition by 5-fluorotryptophan. The experiment was preformed as described for Fig. 4 except 5-fluorotryptophan was added to a final concentration of 4.5×10^{-3} M. Km for the uninhibited reaction=0.91×10^{-3} M; for the presence of inhibitor=1.8×10^{-4} M.

In general, that analogues of biosynthetic intermediates act as growth inhibitors is due to their inhibitory effect on the synthesis or activity of biosynthetic enzymes, while they are unable to substitute for the substrates on growth. Because tryptophan synthetase still has 50% activity under the presence of 5-fluorotryptophan (1 mg/ml), it appears that 5-fluorotryptophan

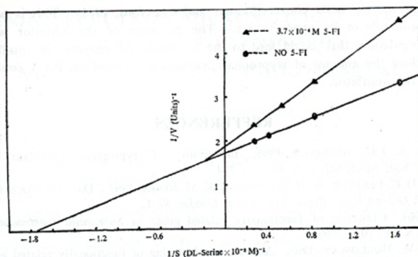


Fig. 6. Kintics of inhibition by 5-fluoroindole. Serine is the limiting substrate, indole is present at optimal concentration (2.5×10^{-4} M). 5-Fluoroindole was added to a final concentration of 3.7×10^{-4} M. The data were also subjected to linear regression analysis (Seteel & Torrie, 1960). Km for the uninhibited reaction$=5.6\times10^{-3}$ M; for the presence of inhibitor$=9.2\times10^{-3}$ M.

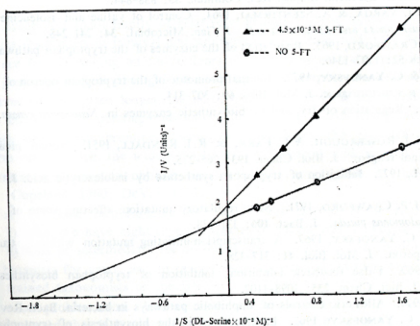


Fig. 7. Kinetics of inhibition by 5-fluorotryptophan. The experiment was performed as described for Fig. 6 except 5-fluorotryptophan was added to a final concentration of 4.5×10^{-3} M. Km for the uninhibited reaction$=5.6\times10^{-3}$ M; for the presence of inhibitor$=1.2\times10^{-2}$ M.

does not inhibit the growth of parental strain completely (Fig. 2). If 5-fluoroindole, which is a strong growth inhibitor to wild type PA-1, exerts its inhibition through the conversion to 5-fluorotryptophan, then its concentration would be too low (100 μg/ml) to inhibit cell growth. This suggests that 5-fluoroindole itself can act as an inhibitor or can be converted to the compounds other than 5-fluorotryptophan to show growth inhibition.

5-FIR of *P. putida*, which accumulates anthranilate, shows that mutation may occur at the

regulatory site for the trp ABD cluster (Maurer and Crawford, 1971). This is possibly the site at which 5-fluoroindole or its products act. The presence of the inhibitor would alter the mechanism of regulation, this could lead to the synthesis of enzymes in insufficient amounts which would reduce the amount of tryptophan synthetase. Therefore, PA-1 could not grow in the presence of this inhibitor.

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