Site of Action of Growth Inhibitory Tryptophan Analogues in *Catharanthus roseus* Cell Suspension Cultures

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Fourteen tryptophan analogues have been characterized with regard to their growth inhibitory effects on *Catharanthus roseus* cell cultures, their inhibitory effect on anthranilate synthetase, their incorporation into protein and their substrate affinity to tryptophan decarboxylase. The toxicity of 12 of the analogues was directly correlated with their inhibitory effect on anthranilate synthetase activity. None of the analogues was efficiently incorporated into protein. Only 4 of the analogues (4-methyl-, 4-fluoro-, 5-fluoro- and 5-hydroxytryptophan) were good substrates for tryptophan decarboxylase while all other analogues were rather poor substrates or were not converted at all. Alpha-methyltryptophan was a competitive inhibitor of tryptophan decarboxylase.

Introduction

Little has been done to develop positive selection systems to screen for plant cell cultures with an increased capability to synthesize distinct metabolites. One reason for this is that the biochemical alterations necessary for increased biosynthesis of metabolites (*e.g.* secondary metabolites) are often unknown. Another reason is that the sites of action of possible selective agents are often not completely understood. The great value of positive selection systems for highly productive plant cells has already been demonstrated. The biotin levels of *Lavandula vera* strains selected on toxic concentrations of pimelic acid were increased up to 5-fold [1]. The selection process yielded cell strains with a higher capacity to detoxify pimelic acid to biotin. Cell lines resistant to *p*-fluorophenylalanine (PFP) were often found to accumulate increased levels of phenolic compounds [2–4]. The higher accumulation of caffeoyl putrescine by the PFP-resistant cell line TX4 was due to enhanced activities of the corresponding biosynthetic enzymes [5]. Phenylalanine ammonia-lyase, the first enzyme of the biosynthetic sequence of phenylpropanoids, detoxifies L-PFP to *p*-fluorocinnamic acid derivatives [6]. The better capability of TX4 cells to detoxify L-PFP contributed at least partly to the resistance [6].

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The increased formation of indole alkaloids in cell cultures of *Catharanthus roseus* [7] and *Peganum harmala* [8] by so called "induction media" [9] was accompanied by greatly enhanced activities of tryptophan decarboxylase (TDC). This enzyme diverts the primary metabolite tryptophan into the secondary pathway of indole alkaloids. Tryptophan decarboxylase is at least one of the limiting factors of alkaloid biosynthesis in these systems. A highly productive cell line should be endowed with high TDC activity. Therefore, it seems to be worthwhile to develop a selection system for cell strains with increased activities of TDC. Some of them may also accumulate increased levels of alkaloids. Our aim was therefore to find tryptophan analogues which are growth inhibitory in the micromolar range and which are decarboxylated by TDC. This led us analyze the mode of action of 14 commercially available tryptophan analogues with *Catharanthus roseus* cell suspension cultures.

Materials and Methods

Cell cultures

Cell suspension cultures of *Catharanthus roseus* (L.) G. Don were maintained in MX-medium (MS-medium [10] supplemented with 2 µM 2,4-dichlorophenoxyacetic acid) and subcultured every two weeks as described [11]. Induction of tryptophan decarboxylase (TDC) was achieved by transferring 14-days-old cells into the 10-fold volume of a solution of 8% sucrose for 2 days.

Toxicity tests

The test for the growth inhibitory effects of the tryptophan analogues and the reversal of inhibition by tryptophan were performed in 50-ml-Erlenmeyer with 25 ml of MX-medium containing varying concentrations of the analogue. The inoculum was 0.3 g fresh mass. After 10 days the increase of cell mass was measured and related to the control.

Extraction of enzymes

The TDC extract was prepared according to Knobloch *et al.* [7] using cells cultivated in 8% sucrose for 2 days. Anthranilate synthetase (AS) was prepared according to Widholm [12] but the extracted protein was precipitated with ammonium sulfate prior to the Sephadex G-25 step.

Enzyme assays

The substrate specificity of the TDC was measured according to Gibson *et al.* [13] with slight modifications. 100 µl substrate solution containing 50 mM Tris-HCl (pH 9.0), 10 mM 2-mercaptoethanol, 0.8 mM pyridoxal-5-phosphate and the tryptophan analogues in different concentration ranges were mixed with 100 µl enzyme solution and incubated at 30 °C for 60 min. The reaction was stopped with 20 µl 1 N KOH. The tryptamines formed were extracted with ethyl acetate and separated by TLC on silica gel using $CHCl_3$: MeOH:25% NH_3 (5:4:1). The tryptamines were directly measured at 275 nm or, after spraying the plates with 0.1% Ninhydrin solution, at 395 nm, using a Shimadzu TLC-scanner. The calibration curve was prepared using tryptamine. The K_m-values were calculated from Lineweaver and Burk plots. The feedback effect of the different tryptophan analogues on the activity of the anthranilate synthetase was measured according to Widholm [12] and related to the control.

Incorporation of the analogues into protein

2.5 g cells were added to 70 ml growth medium supplemented with 10 µM of the tryptophan analogue. After 3 days the cells were harvested and thrown into liquid nitrogen. The shock-frozen cells were then extracted with 5 ml H_2O (Ultra Turrax) and centrifuged. The supernatant was treated with the 3-fold volume of a saturated $(NH_4)_2SO_4$-solution, stirred for 1/2 h and centrifuged. The pellet was taken up in 2.5 ml H_2O and eluted from a Sephadex PD-10 column with 3.5 ml H_2O. In controls, the analogue was added to the culture just before the harvest. The protein solutions were hydrolyzed with $Ba(OH)_2$ [14] in ampoules in an autoclave at 121 °C for 24 h. The $Ba(OH)_2$ solution was brought to pH 6 with 2 N H_2SO_4 and centrifuged. The supernatant was evaporated to dryness, taken up in 0.5 ml H_2O and analyzed for tryptophan analogues by HPLC (Lichrosorb RP-18-5 µ, 250 × 4 mm, precolumn RP-2-30 µ, MeOH: 10 mM sodium acetate, 14:86; flow rate 1 ml/min, detection at 280 nm). This system separates the analogues from tryptophan. As positive control, the incorporation of [3-^{14}C]-L-tryptophan (10 µM) into protein was measured under the same experimental conditions.

Results

Toxicity of tryptophan analogues

Fourteen commercially available tryptophan analogues and tryptophan itself were tested for their toxicity. As a measure of toxicity fresh mass increases in relation to analogue concentrations were determined. Results obtained with several methylated analogues are shown in Fig. 1. To characterize and to compare the toxicities of the tested analogues the highest non-inhibitory, the 50%-inhibitory and the lowest complete-inhibitory level are given in Table I. According to this the most toxic tryptophan derivative is α-methyltryptophan,

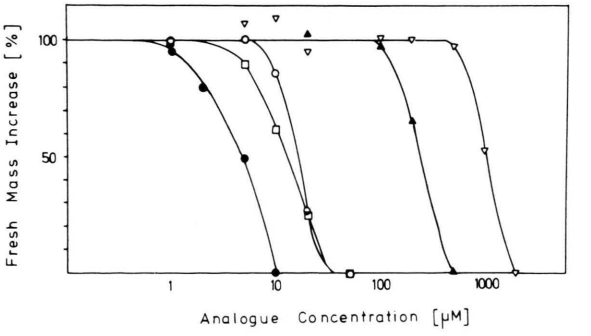

Fig. 1. Growth inhibition of *Catharanthus roseus* cell suspension cultures by different methylated tryptophan analogues. △ 1-methyl-tryptophan; ○ 4-methyl-tryptophan; □ 5-methyl-tryptophan; ▲ 6-methyl-tryptophan; ● α-methyl-D,L-tryptophan.

Table I. Growth inhibition of *Catharanthus roseus* cells by tryptophan and tryptophan analogues.

Inhibitor	Non inhibitory level (growth > 90%)	50% inhibitory level [mM]	Complete inhibitory level (growth < 5%)
L-Tryptophan	0.2	1	5
D-Tryptophan	–	0.5	5
α-Methyltryptophan	0.001	0.005	0.01
N-Methyl-L-tryptophan	< 0.01	0.05	> 2
1-Methyltryptophan	0.5	1.0	2.0
4-Methyltryptophan	0.008	0.015	0.03
5-Methyltryptophan	0.005	0.012	0.03
6-Methyltryptophan	0.1	0.23	0.5
7-Methyltryptophan	< 0.01	0.02	0.2
4-Fluorotryptophan	0.002	0.011	0.04
5-Fluorotryptophan	0.008	0.014	0.02
6-Fluorotryptophan	0.01	0.025	0.03
5-Hydroxytryptophan	–	–	0.01
5-Methoxytryptophan	0.01	0.040	0.12
5-Benzyloxytryptophan	0.2	0.7	1.0
7-Azatryptophan	0.2	0.8	1.2

the least toxic are 5-benzyloxy-, 7-aza- and 1-methyltryptophan. The latter and also N-methyltryptophan were completely inhibitory only in the millimolar range. Six of the analogues have recently also been tested for their toxicity to carrot and tobacco cell cultures [15]. Those data compare well with the results found with *Catharanthus* cultures. From the point of view of toxicity, only 10 of the 14 analogues seemed to be good candidates for the proposed selection scheme.

The toxic effect of a true analogue should be reversible or at least be relieved by the addition of L-tryptophan. The relief of growth inhibition of the tryptophan analogues by L-tryptophan is given in Table II.

Except for 5-benzyloxytryptophan all tested compounds can be regarded as analogues. In some cases not only the growth inhibition but also the "toxic" effect of higher concentrations of L-tryptophan were reversed.

Inhibition of anthranilate synthetase

Some tryptophan analogues have been shown to inhibit anthranilate synthetase, causing a depletion of L-tryptophan [15]. The inhibiting effect of tryptophan and the fourteen analogues on anthranilate synthetase was tested. Inhibition curves of L- and D-tryptophan and different methylated analogues are shown in Fig. 2. Data characterizing all tested compounds were compiled from such curves and are listed in Table 3. From the data for L- and DL-tryptophan, 5-hydroxy-L- and 5-hydroxy-DL-tryptophan and the curve for D-tryptophan (Fig. 2) it is clear that the D-forms have nearly no inhibiting effect on the AS activity and cannot be regarded as L-tryptophan analogues. Therefore, when DL-mixtures were used, the active concentrations were half of the given concentrations. Alpha-, 4- and 5-methyltryptophan and 4-, 5- and 6-fluorotrypto-

Table II. Effect of L-tryptophan on the growth inhibition caused by the tryptophan analogues. The percentage of growth inhibition caused by the addition of L-tryptophan to the controls are given in brackets.

Analogue added	Concentration [mM]	Increase of fresh mass related to control (%) L-tryptophan added 0 mM	0.5 mM
None	–	100	100 (84)
1-Methyltryptophan	1.0	78	110
4-Methyltryptophan	0.09	0	94
5-Methyltryptophan	0.09	0	97
6-Methyltryptophan	1.0	0	45
7-Methyltryptophan	1.0	0	36
α-Methyltryptophan	0.03	0	59
N-Methyl-L-tryptophan	1.0	41	153
4-Fluorotryptophan	0.12	0	62
5-Fluorotryptophan	0.06	0	58
6-Fluorotryptophan	0.06	0	81
5-Hydroxytryptophan	0.30	0	133
5-Methoxytryptophan	0.36	3	69
5-Benzyloxytryptophan	1.0	10	0
7-Azatryptophan	1.0	0	75

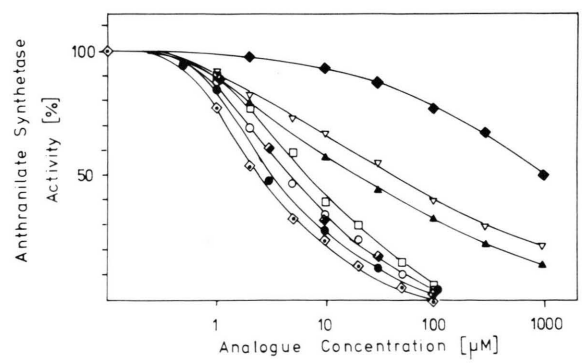

Fig. 2. Inhibition of anthranilate synthetase in extracts from *Catharanthus roseus* cell suspension cultures by tryptophan and its methylated analogues. ⊡ L-tryptophan; ■ D-tryptophan; ▬ D,L-tryptophan. Other signs see Fig. 1.

Table III. Inhibition of anthranilate synthetase in extracts from *Catharanthus roseus* cell suspension culture.

Inhibitor	Non inhibitory level (activity > 90%)	50% inhibitory level [μM]	Complete inhibitory level (activity < 5%)
L-Tryptophan	0.4	2.5	50
DL-Tryptophan	0.8	5.0	90
α-Methyl-DL-tryptophan	0.8	3.0	60
N-Methyl-L-tryptophan	4.0	20	300
1-Methyl-DL-tryptophan	1.0	40	>3000
4-Methyl-DL-tryptophan	0.8	5.0	90
5-Methyl-DL-tryptophan	0.9	7.0	100
6-Methyl-DL-tryptophan	1.0	20	3000
7-Methyl-DL-tryptophan	4.0	20	300
4-Fluoro-DL-tryptophan	1.0	7.0	200
5-Fluoro-DL-tryptophan	0.6	3.0	40
6-Fluoro-DL-tryptophan	0.5	3.0	30
5-Hydroxy-L-tryptophan	4.0	25	400
5-Hydroxy-DL-tryptophan	8.0	50	700
5-Methoxy-DL-tryptophan	3.0	15	300
5-Benzyloxy-DL-tryptophan	2.0	10	500
7-Aza-DL-tryptophan	30	300	2000

phan inhibited the AS in the same concentration range as tryptophan itself. 1- and 6-methyltryptophan and 7-azatryptophan were clearly less inhibitory. They inhibited AS activity completely only in the millimolar range.

If one compares the data of Table I with those of Table III correlation can be seen between the toxicity of an analogue and its inhibiting effect on the AS. In Fig. 3 the values for a complete growth inhibition are plotted against the concentration that inhibited the AS activity by 50%. The linear regression line of the double log plot shows a high coefficient of determination ($r^2 = 0.90$) indicating a possible correlation between toxicity and AS inhibition. The data for 5-hydroxytryptophan and 5-benzyloxytryptophan were not used for the calculation of the linear regression. The values for both analogues deviate from the other. 5-Hydroxytryptophan is clearly more toxic than one can estimate from the AS inhibition data. 5-Benzyloxytryptophan was excluded because it showed no antimetabolite behavior in the reversion experiments (Table II). It is less toxic than expected from the AS inhibition data.

Incorporation of tryptophan analogues into protein

The toxicity of the tryptophan analogues may also result from an incorporation of the analogue into protein. However, none of the alkaline protein hydrolysates contained higher levels of the analogues than the controls. Therefore it is concluded that the analogues were not incorporated into protein under the experimental conditions. After feeding 5-methyltryptophan-[$^{14}C-CH_3$], the only commercially available radioactive labelled tryptophan analogue, to *Catharanthus roseus* cell cultures, no radioactivity was found in the protein fraction while this was the case when labelled L-tryptophan was added to the cultures.

Substrate specificity of the tryptophan decarboxylase

Amino acid analogues may also be metabolized, possibly to less toxic compounds. Since 5-methyltryptamine is roughly 100 times less toxic to *Catharanthus* cells than DL-5-methyltryptophan, the ability of TDC to decarboxylate the 14 analogues was examined. Phenylalanine and tyrosine were also tested as substrates for this enzyme. The K_m values calculated from Lineweaver-Burk plots are given in Fig. 4. With the exception of tryptophan, 5-hydroxytryptophan and N-methyltryptophan all amino acids tested were DL-mixtures, but K_m values were always related to the L-form since D-tryptophan was not a substrate of TDC. The substrate

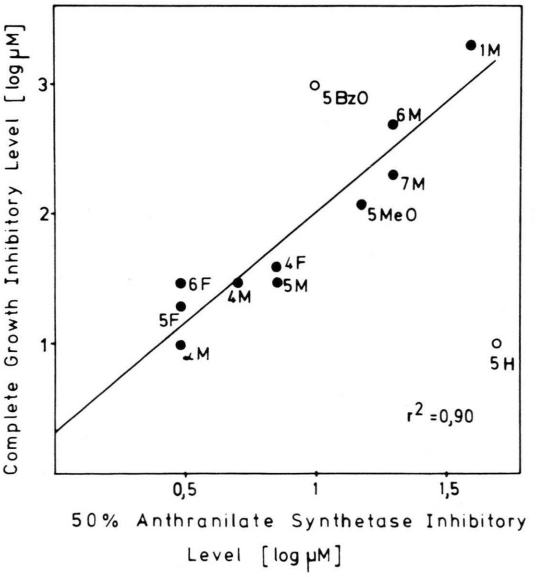

Fig. 3. Correlation between inhibition of growth and anthranilate synthetase activity by several tryptophan analogues (regression line calculated by the values of black signs).

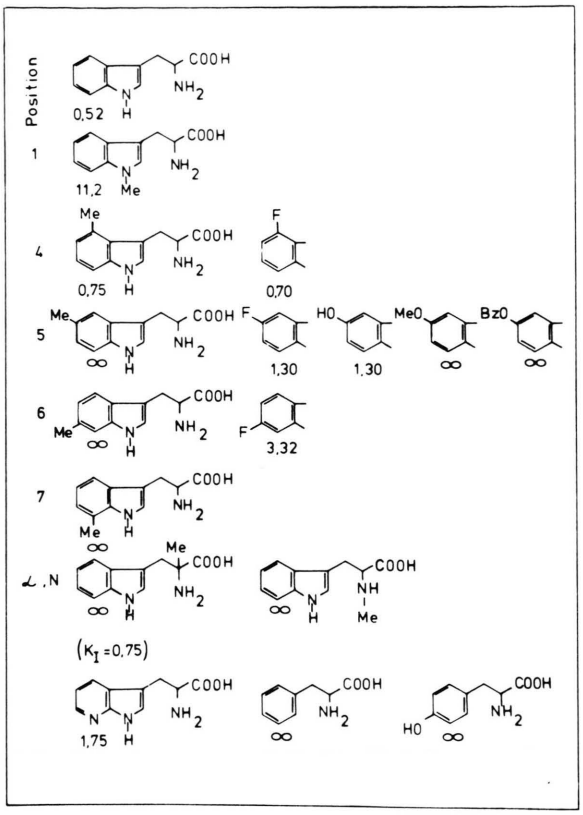

Fig. 4. Substrate specificity of tryptophan decarboxylase from *Catharanthus roseus* cell suspension culture (Figures give the K_m values, calculated for the L-form (mM); ∞ no turnover measurable).

affinity changed strongly with the substitution pattern at the aromatic ring. Alteration at the 4-position of the tryptophan molecule evidently had the lowest effect on the activity of such substrates with TDC (Fig. 4). While the introduction of a fluoro or a hydroxy group at the 5-position gave still reasonable substrates for TDC, the introduction of a methyl group already prevented the enzymatic decarboxylation of this molecule (Fig. 4). Alterations at all other positions gave also rise to molecules which were rather poor substrates. The identity of the tryptamine analogues produced was checked by GC-MS. Alpha-methyltryptophan was found to be a competitive inhibitor with K_I = 0.75 mM. Alphamethyl amino acids in general have been shown to be competitive inhibitors of the corresponding amino acid decarboxylases [16]. Phenylalanine and tyrosine were not decarboxylated by the enzyme extract indicating that this inducible decarboxylase in *Catharanthus roseus* was not a general aromatic amino acid decarboxylase.

Discussion

Possible toxic mode of actions of amino acid analogues are false feedback inhibition of the synthesis of the natural amino acid [15] and/or incorporation of the analogue into protein leading to inactive enzymes [17]. When the artificial amino acid acts as a true analogue of the natural amino acid then the growth inhibitory effect of the analogue can be relieved by the addition of the corresponding natural amino acid. Except for the rather nontoxic 5-benzyloxytryptophan, all tested tryptophan analogues can be regarded as true analogues mimicking the action of tryptophan. Our investigation confirms and extends the data presented by Widholm [16] with tobacco and carrot cultures. The toxic effects of *p*-fluorophenylalanine are only poorly relieved by L-phenylalanine [2]. For compounds like PFP or 5-benzyloxytryptophan therefore the mode of toxicity is not related to the metabolism of the corresponding natural amino acid and it is questionable to call them analogues.

In our experiments the toxicity of an analogue and its inhibitory effect on the AS activity were well correlated (with the exception of 5-benzyloxytryptophan and 5-hydroxytryptophan). That leads to conclude that false feedback inhibition preventing further synthesis of L-tryptophan for protein biosynthesis is the important factor for the toxic action of the tryptophan analogues in the *Catharanthus roseus* cultures, as well as in tobacco and carrot cultures [15]. It is evident that a possible toxic effect of the analogue by incorporation into protein can be excluded or at least ignored.

Plant cell cultures resistant to 5-methyltryptophan have been described. It has been shown that the anthranilate synthetase of many of these resistant cell cultures was less sensitive to feedback inhibition, which resulted in the cells accumulating higher levels of L-tryptophan [18–21]. Since the toxicity of the analogues is directly related to their inhibitory effect on AS activity it is likely that with most of the tested tryptophan analogues one will select resistant cell lines with increased levels of endogenous L-tryptophan and likely an altered AS.

Beside variants which became resistant due to an altered uptake of the toxic analogue [2, 20] one should be able to select cells with a higher capacity to detoxify the analogue. From this study it is clear that 4-methyl-, 4-fluoro, 5-fluoro- and 5-hydroxy-tryptophan should be the best selective agents to find this type of variant. These variants should have increased TDC activity and some of these 4-methyl-, 4-fluoro-, 5-fluoro- and 5-hydroxy-tryptophan resistant cell lines with increased TDC activity might also have an increased capacity to synthesize indole alkaloids. The biochemical characterization of such resistant cell lines is given in the following paper [22].

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