# Selection of Cell Lines of *Catharanthus roseus* with Increased Tryptophan Decarboxylase Activity

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Z. Naturforsch. **38 c**, 916–922 (1983); received September 19, 1983

*Catharanthus roseus*, Cell Suspension Cultures, Tryptophan Analogues, Resistant Cell Lines, Tryptophan Decarboxylase, Indole Alkaloids

Cell lines of *Catharanthus roseus* were selected for resistance to 4-methyltryptophan (4-MT) in order to get strains with increased activities of tryptophan decarboxylase (TDC). This enzyme may exert a decisive regulatory control in indole alkaloid biosynthesis. 4-Methyltryptophan is a substrate for TDC and is detoxified by this enzyme. All 4-MT resistant cell lines showed increased TDC activity (3-to 10-fold) and higher accumulation of tryptamine (3- to 5-fold). Two of these cell lines with increased TDC activity accumulated indole alkaloids in the growth medium, whereas in wild type cultures and other resistant cell lines this was not the case. All the cell lines obtained were not only resistant to 4-MT (20- to 70-times more than wild type cells) but were also cross-resistant to other tryptophan analogues which are not metabolized by TDC. Therefore the resistance was not only due to a better detoxification of the analogue. The selected cell lines also contained 2 to 6 times higher levels of L-tryptophan but the feedback sensitivity of the anthranilate synthetase of the resistant cells was not altered.

## Introduction

The biotechnological exploitation of plant cell cultures for the production of natural products is hampered by the fact that plant cell cultures very often accumulate only small amounts of the desired metabolites. Today two strategies are widely recommended to stimulate the productivity of a cell culture – analytical screening and/or media optimization. By using sensitive analytical tools one can detect variant cells synthesizing or accumulating higher levels of secondary compounds in the heterogeneous wild type population [1–3]. These highly productive variant cells have been recultivated separately and have sometimes resulted in high producing, stable cell lines after repeated cloning [2, 4, 5]. However, often high producing cell lines derived from analytically screened cell colonies revert to low production after a few subcultures [1, 3, 6]. Even if cell lines were derived from analytically characterized single cells of a suspension culture there was no correlation between the productivity of the mother cell and the derived culture [3].

Growth and production of secondary metabolites are often antagonistic processes in a cell culture. Tissue culture media are defined for optimal growth but some of the constituents of growth media repress secondary metabolism [7, 8]. Only after depletion of these constituents or after transfer to special "induction" or "production" media [8] is secondary metabolism turned on [6–12]. Normally, however, growth is greatly reduced in these media. While the specific yield (mg/g) may be high, the total yield (mg/l × d) may be rather low.

Despite the indisputable value of these two techniques we are working on an additional technique – the biochemical selection of highly productive cell lines. It has been shown that *p*-fluorophenylalanine resistant cell lines often accumulate increased levels of phenolic compounds [13, 14]. A biochemical explanation as well as the advantages and disadvantages of this system have been recently discussed [15]. A good example of the kind of strains biochemical selection may provide is the caffeoyl putrescine producing tobacco cell line TX4 [16].

In analogy to our work with *p*-fluorophenylalanine resistant cells we have tried to establish a similar system for *Catharanthus roseus*. Analytically screened *Catharanthus* cells have rather unstable production characteristics [1] and the strains often produce indole alkaloids only after transfer into a special induction medium [7]. Investigations on the formation of indole alkaloids in *Catharanthus roseus*

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[17] showed that the activity of the tryptophan decarboxylase (TDC) could be correlated to the product level. TDC activity seemed to be at least one limiting factor for the alkaloid biosynthesis. By contrast, the next enzyme of this pathway, strictosidine synthase, was shown to be constitutive [17]. We tried therefore to select cell lines with higher TDC activity. Among these we expected to find a few lines in which not only TDC activity but also other regulatory enzymes of indole alkaloid biosynthesis were constitutively expressed. Such cell lines should produce indole alkaloids in the growth medium. In the preceding paper we demonstrated that some tryptophan analogues can be converted by TDC to the less toxic tryptamine analogues [18]. 4-Methyltryptophan and 4-fluorotryptophan were regarded as the best analogues to select for the wanted trait. Here we report the characterization of some 4-methyltryptophan resistant cell lines.

## Materials and Methods

*Cell cultures and selection of resistant lines*

Cell cultures of *Catharanthus roseus* (L.) G. Don were maintained as described [18]. 4-Methyl-DL-tryptophan (4-MT) resistant lines were selected in liquid MX medium [18] from two wild types CP-3A and CP-3B. CP-3A accumulated only tryptamine after transfer to 8% sucrose, while CP-3B also formed indole alkaloids in this medium. For one selection series ~ 25 Erlenmeyer flasks were inoculated with 0.7 g fresh mass/70 ml medium. Growth was observed in some of them after 2 to 4 months. 4-MT resistant lines derived from CP-3A were labelled with AM and a number. Strains with labels of one figure were selected against 0.2 mM 4-MT, lines with two figures twice, first against 0.2 mM, than against 1.0 mM 4-MT. The lines that derive from CP-3B are labelled with BM. Resistant lines were maintained with and without the selective concentration of 4-MT.

*Resistance tests*

Tests for resistance were carried out in 50-ml-Erlenmeyer flasks with 25 ml of MS-medium containing varying concentrations of the analogues. The inoculum was 0.3 g (fresh mass). The increase of cell mass was measured after 10 days and related to the control without any analogue.

*Enzyme extraction and assays*

The tryptophan decarboxylase (TDC) extract was prepared according to [17] and the anthranilate synthetase (AS) extract according to [19], but in addition the protein was precipitated with ammonium sulfate prior to the Sephadex G 25 step. The enzyme assays have been described previously [18], but TDC was measured in a radiometric test using [3-$^{14}$C]-L-tryptophan as substrate. After the reaction was stopped with KOH, the radioactivity extractable with ethyl acetate was measured in a liquid scintillation counter against blanks (inactivated enzyme). Protein content of the extracts was determined by the biuret method [20].

*Determination of metabolites*

100 mg freeze dried cells were extracted with 10 ml 70% ethanol (v/v) using a vortex mixer and centrifuged. An aliquot of the supernatant was evaporated, resuspended in water and extracted under alkaline conditions with dichloromethane. The aqueous phase was used to determine tryptophan [21]. The organic phase was separated by TLC on silica gel using xylene:2-butanone:diethylamine (150 + 75 + 4.5) and $CHCl_3$:MeOH:25% $NH_3$ (10 + 4 + 1) as solvents. Ajmalicine and tryptamine were quantified directly by their absorbance at 280 nm using a Shimadzu TLC scanner.

## Results

*Resistance of the selected cell lines*

After selection, the cells were subcultured for a number of growth cycles with and without 4-MT. Their resistance against different amino acid analogues was then tested. Table I gives the complete inhibitory level of the two wild type cultures (CP-3A; CP-3B) and the selected lines. The data show that all cell lines tested are not only resistant to the selective agent 4-MT but also to other tryptophan analogues. The resistance against 5-FT is even higher than the resistance of the most resistant cell lines selected directly on 5-FT. The line BM2 had also a slightly elevated resistance against unrelated analogues like *p*-fluorophenylalanine (PFP) and aminoethylcysteine (AEC). A resistance against 5-MT, PFP and AEC cannot be explained by a higher detoxification by TDC, because these analogues are not substrates of this

Table I. Resistance of cell lines of *Catharanthus roseus* selected against 4-methyltryptophan to different amino acid analogues.

| Line | Selective concentration [mM] | Growth cycles +/−[a] | Complete inhibitory level (growth < 5% of control) [mM] | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | 4-MT | 5-MT | 4-FT | 5-FT | PFP | AEC |
| CP-3A/CP-3B | – | – | 0.03 | 0.03 | 0.04 | 0.02 | 0.01 | 0.01 |
| AM1 | 0.2 | 4/3 | 1.5 | 0.7 | 0.1 | 0.1 | – | – |
| | | 12/10 | 1.0 | – | – | – | – | – |
| AM2 | 0.2 | 4/3 | 1.5 | 1.0 | 0.2 | 0.3 | – | – |
| | | 4/19 | 0.5 | – | – | – | – | – |
| AM3 | 0.2 | 4/3 | 2.0 | 1.5 | 0.2 | 0.1 | – | – |
| | | 4/19 | 0.5 | – | – | – | – | – |
| AM1.1 | 1.0 | 2/9 | 1.1 | – | – | – | – | – |
| AM4.1 | 1.0 | 3/3 | 2.0 | 2.5 | 0.2 | 0.5 | – | – |
| | | 11/10 | 2.0 | – | – | – | – | – |
| BM2 | 0.2 | 10/0 | 0.5 | 0.2 | 0.05 | 0.05 | 0.05 | 0.02 |
| | | 0/16 | 0.05 | 0.05 | 0.02 | 0.01 | 0.02 | 0.01 |

[a] +/− = with and without 4-MT; MT = methyltryptophan, FT = fluorotryptophan, PFP = *p*-fluorophenylalanine, AEC = aminoethylcysteine.

enzyme. Other resistance mechanisms like a generally reduced uptake of amino acids or – in the case of 5-MT – an increased tryptophan pool, which dilutes the toxic effect of the analogue have to be considered. The data of the table also show that there is often a decrease in the resistance, when the lines are cultivated without 4-MT for a longer period of time. The loss of resistance was more pronounced in lines that had been grown for only a few subcultures in the presence of 4-MT. This is especially evident in the BM2 line. An aliquot of cells that were not subcultured in 4-MT containing media after selection formed on analogue sensitive population again after 16 growth cycles. Lines that were selected against higher concentrations and had been grown in the presence of the analogue for more subcultures seemed to be more stable. Thus, the line AM 4.1, which was selected and grown for 11 growth cycles in the presence of a high concentration of 4-MT (1.0 M), showed no loss of resistance after another 10 growth cycles without the analogue. To prevent a decreasing resistance all lines were normally maintained in the presence of the selective agent.

As shown in Fig. 1 for the BM2 line, resistant cell strains in general grew more slowly, especially when the analogue was present in the medium. Ultimately, however, the same amount of cell mass was reached.

*Analytical characterization*

Table II shows the tryptophan, tryptamine and ajmalicine content and the TDC activity of the wild type and the selected lines, after they had been grown for 14 d in the normal growth medium. The selected lines showed a 2 to 6-fold increased level of tryptophan compared to wild type cells. All lines

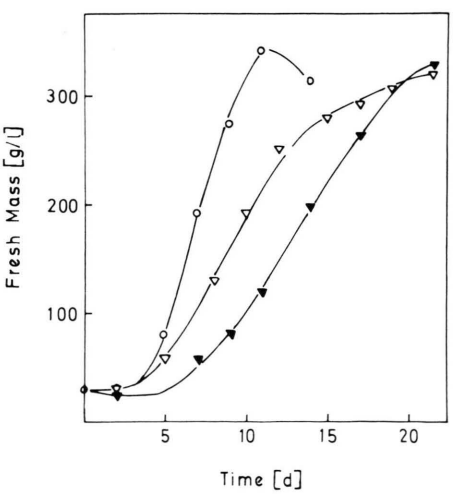


Fig. 1. Growth of wild type CP-38 (○—○) and the (▽—▽) selected line BM2 in MS medium, and growth of AM2 in the presence of 4-MT (▼—▼). The data for BM2 were received from cells grown for 3 cycles in the absence of 4-MT.

Table II. Tryptophan, tryptamine and ajmalicine content and TDC activity of 14d old cells of different cell lines of *Catharanthus roseus* selected against 4-methyltryptophan (contents related to dry mass, TDC activity to protein).

| Line | Selective concentration [mM] | Growth cycles +/−[a] | Tryptophan [mg/g] | Tryptamine [mg/g] | Ajmalicine [mg/g] | TDC [nkat/g] |
|---|---|---|---|---|---|---|
| CP-3A | – | | 0.34 ± 0.12 | 0.27 ± 0.02 | 0.0 | 10 ± 5 |
| CP-3B | – | | 0.16 ± 0.01 | 0.28 ± 0.04 | 0.0 | 3 ± 2 |
| AM1 | 0.2 | 4/3 | 1.07 ± 0.03 | 0.69 ± 0.01 | 0.0 | 28 ± 1 |
| | | 12/1 | 0.61 ± 0.01 | 0.86 ± 0.06 | 0.0 | 36 ± 5 |
| | | 2 | 0.44 ± 0.07 | 0.93 ± 0.06 | 0.0 | 27 ± 4 |
| | | 3 | 0.47 ± 0.19 | 0.85 ± 0.03 | 0.0 | 30 ± 1 |
| AM2 | 0.2 | 4/3 | 1.63 ± 0.12 | 1.42 ± 0.21 | 0.0 | 31 ± 5 |
| | | 14 | 0.55 ± 0.08 | 0.61 ± 0.06 | 0.1 | – |
| AM3 | 0.2 | 4/3 | 1.86 ± 0.10 | 1.47 ± 0.18 | 0.0 | 56 ± 13 |
| | | 14 | 0.54 ± 0.07 | 0.65 ± 0.05 | 0.0 | – |
| AM1.1 | 1.0 | 2/1 | 0.87 ± 0.16 | 1.52 ± 0.01 | 0.0 | 25 ± 5 |
| | | 2 | 0.74 ± 0.04 | 0.72 ± 0.09 | 0.0 | 29 ± 14 |
| | | 3 | 1.07 ± 0.04 | 0.78 ± 0.05 | 0.0 | 20 ± 4 |
| AM4.1 | 1.0 | 3/3 | 0.77 ± 0.15 | 0.88 ± 0.55 | 0.0 | 34 ± 3 |
| | | 11/1 | 0.99 ± 0.19 | 1.44 ± 0.08 | 0.0 | 28 ± 2 |
| | | 2 | 0.67 ± 0.56 | 1.40 ± 0.38 | 0.0 | 44 ± 17 |
| | | 3 | – | 1.28 ± 0.05 | 0.0 | 30 ± 1 |
| BM1 | 0.2 | 0/1 | 0.36 ± 0.08 | 1.38 ± 0.33 | 0.40 ± 0.04 | 26 ± 4 |
| BM2 | 0.2 | 0/1 | 0.46 ± 0.09 | 1.09 ± 0.27 | 1.44 ± 0.16 | – |
| | | 3 | – | 0.85 ± 0.01 | 0.50 ± 0.05 | 31 ± 9 |
| BM3 | 0.2 | 0/1 | 0.19 ± 0.04 | 1.64 ± 0.30 | 0.0 | – |

[a] +/− with and without 4-MT.

also showed the desired trait, increased activity of the TDC. These higher activities were combined with higher levels of tryptamine, but in the CP-3A derived lines no alkaloids were detectable. Only two lines, which were derived from the CP-3B wild type, were found to accumulate alkaloids, mainly ajmalicine. Neither of the two wild type cultures was able to accumulate alkaloids in the growth medium. By transferring the cells to a solution of 8% sucrose [7] only CP-3B cells could be induced to produce tryptamine and alkaloids, while CP-3A only accumulated tryptamine. The line BM2 formed levels of ajmalicine in the normal growth medium that were already higher than the values that were reached by inducing the wild type.

*Feedback sensitivity of the anthranilate synthetase*

Although there seem to be some changes in the tryptophan pools of the selected lines, no alteration of the feedback sensitivity to tryptophan (Fig. 2) and 4-MT (not shown) had occurred. To be sure that the tested cultures had not lost their resistance, these tests were also done with cells which were grown always in the presence of 4-MT.

*Kinetic studies*

Because the growth behavior of wild types and selected lines can be different (see Fig. 1), a one point analysis only gives preliminary results. Therefore Figs. 3 and 4 show kinetic studies of the AM1 and BM2 line in comparison with their wild types. In both cases there was a clear difference between the wild type and the derived resistant line. The AM1 line (Fig. 3) reached a maximum TDC activity of 60 nkat/g protein at day 11, the wild type CP-3A only 20 nkat at day 8. The different TDC activities were reflected in a different level of tryptamine accumulation. The AM1 line reached up to 0.9 mg/g dry weight at day 17, the wild type cells only 0.3 mg at day 8. As mentioned before both cultures did not accumulate alkaloids. The BM2 line (Fig. 4) had a maximum TDC activity of 100 nkat/g at day 8, whereas the wild type activities always stayed below 5 nkat/g. Tryptamine was

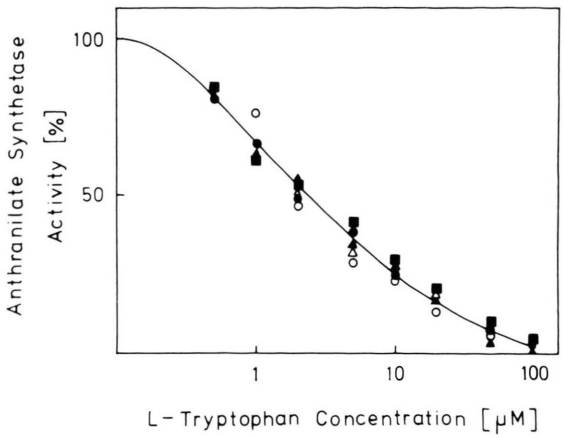


Fig. 2. Inhibition of anthranilate synthetase (AS) extracted from wild type and 4-MT resistant cell lines of *Catharantus roseus* by L-tryptophan (CP-3A: △, CP-3B: ○, AM1: ▲, AM4.1: ■, BM2: ●).

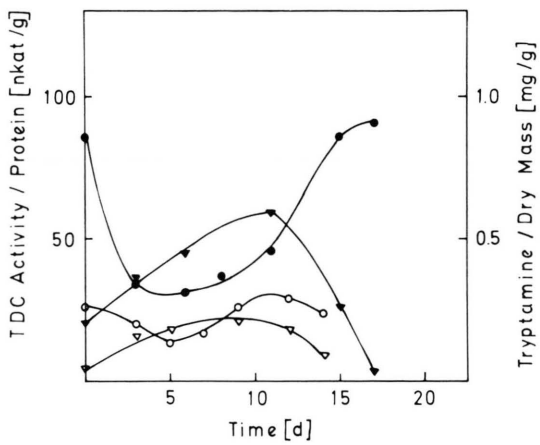


Fig. 3. TDC activity (▽—▽, ▼—▼) and tryptamine (○—○, ●—●) content of the wildtype line CP-3A (open symbols) and the 4-MT resistant line AM1 (12/1) (filled symbols).

accumulated up to 1.0 mg/g, but there also was an accumulation of alkaloids, the ajmalicine concentration reached 0.4 mg/d dry weight at day 17. The wild type showed a maximum tryptamine amount of 0.3 mg/g, but no alkaloids.

## Discussion

As shown in the preceding paper, only a few tryptophan analogues can be detoxified by TDC of *Catharanthus roseus* cultures [18] to rather untoxic tryptamine analogues. These analogue substrates of TDC (4- and 5-fluoro-, 5-hydroxy-, and 4-methyl-

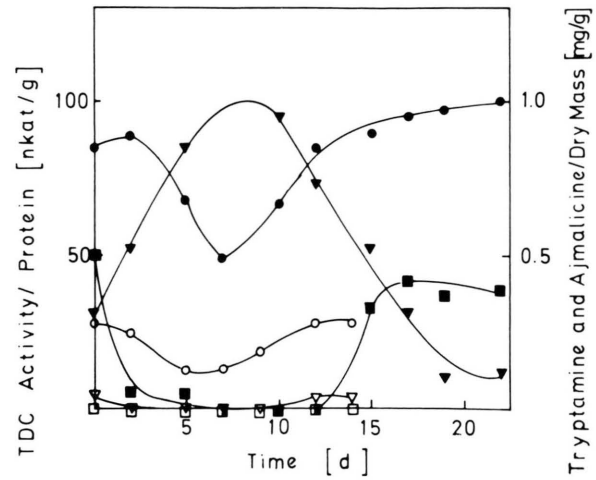


Fig. 4. TDC activity (▽—▽, ▼—▼) and tryptamine (○—○, ●—●) and ajmalicine (□—□, ■—■) content of the wildtype cell line CP-3B (open symbols) and the 4-MT resistant BM2 (0/3) line (filled symbols).

tryptophan) were used to select for cell strains with increased TDC activity. In such resistant lines TDC derived products should be increased. A first analysis of the selected lines showed that the 4-methyltryptophan resistant ones seemed to be the most promising lines with respect to secondary metabolism. With nearly all these lines, however, we observed a loss of resistance when the cells were grown without the selective agent. The loss of resistance may be due to the fact that the 4-MT does not kill the cells, but only inhibits the growth. Even after some subcultures in the presence of the analogue there may be some sensitive cells left which will overgrow the resistant cells in the absence of the analogue. This might be prevented by a prolonged culture of the cells in the presence of the analogue. Another possibility is that only adapted cells were selected that stayed in a certain insensitive state, when the analogue was present and became sensitive again when the analogue was removed from the culture medium [22]. This might be prevented by a second more rigorous selection against an increased concentration of the analogue. The results of the AM4.1 line show that it was thus possible to get a stable line. It was also possible to stabilize the cultures by keeping them under permanent selection pressure, *i.e.* in the permanent presence of the analogue.

It is a well known fact that amino acid analogues can be used as selective agents to establish cell lines

with increased levels of the corresponding free amino acids. Since the biosynthesis of amino acids in higher plants is often feedback controlled, it is reasonable to find a lessened feedback control by the overproducer lines [23, 24]. Recently two groups described cell lines of *Catharanthus roseus* resistant to 5-MT [25, 26]. Tryptophan levels were greatly increased in the resistant lines. While Scott's group reported a lessened feedback control for the anthranilate synthetase of the resistant cell lines [26], our group found no changes for the feedback inhibition pattern of anthranilate synthetase [25]. The selection with other tryptophan analogues inhibiting anthranilate synthetase activity resulted also in resistant cell lines with increased levels of free L-tryptophan. Again in none of the lines tested so far (as shown here for the 4-MT-resistant cell lines; Fig. 2) was the feedback inhibition of anthranilate synthetase reduced in comparison to wild type cells. The highest level of L-tryptophan accumulation were observed in 5-MT resistant cells [23, 24]. Our 4-MT or 5-FT resistant lines had clearly lower levels of L-tryptophan. For increases of this magnitude biochemical alterations may not be necessary. However, our failure to detect an altered anthranilate synthetase may also indicate that other enzymes are involved in the overall control of tryptophan biosynthesis in *Catharanthus roseus.*

The most important aim of this work was to demonstrate that one can select for cell lines with a better capacity to detoxify an amino acid analogue. Since all 4-MT resistant cell lines showed a higher activity of TDC than did wild type, we assume that it is in general possible to devise selection schemes for establishing plant cell cultures with higher activities of detoxifying enzymes. The higher detoxification rate of 4-MT via TDC was of course not the only reason for the overall resistance of the selected cell strains but it surely contributed to the resistance. After feeding of 4-MT to *Catharanthus roseus* cells we detected 4-methyltryptamine in the cell extracts by GCMS analysis (Witte and Sasse, unpublished). The resistant cell lines with its increased levels of TDC had consequently higher levels of tryptamine and in few cases, of ajmalicine, too. 5-Methyltryptophan was not decarboxylated by TDC preparations [18]. Selection against 5-MT therefore did not lead to lines with higher levels of TDC or increased levels of tryptamine or indole alkaloids but led to tryptophan overproducing cell lines [25]. If one uses amino acid analogues not only to select for resistance but also to select for a wanted trait, it would be favourable to know the mode of action of the analogue or how it is metabolized. The different types of resistant variants resulting from selections against 5-MT or 4-MT clearly confirms this statement. Nevertheless, one always has to keep in mind that the resistance may be due to more than one biochemical alteration. Only single cell cloning and screening for the wanted trait may result in resistant cell lines with only one biochemical characteristic altered.

In these experiments, a higher TDC activity did not automatically lead to a higher alkaloid accumulation. The reason may be that we lifted only the regulatory control in the tryptophan derived branch of the biosynthetic pathway but did not select for an altered control of the terpenoid branch, which delivers secologanin. Investigation of the regulatory controls in the biosynthesis of secologanin may give hints how to influence this pathway, too, and how to select cells with higher activities of its key enzymes. It was also interesting to see that only the B-strain in which indole alkaloid biosynthesis is inducible provided resistant strains with constitutively expressed indole alkaloid biosynthesis. The A-strain who had lost its capacity to form indole alkaloids gave only resistant strains with increased tryptamine levels. Since in none of the A-derived resistant strains indole alkaloid was inducible or constitutively expressed we feel that one should use our selection scheme only as an enrichment technique for those traits which are already present in low levels in the wild type. However, a conclusive answer to this question can only be given if large numbers of resistant clones would be analyzed.

In summary, we regard the biochemical selection for higher levels of constitutively expressed TDC as a preselection. An analytical screening of alkaloid producing resistant cell lines is in progress. These biochemically preselected and analytically screened cell lines could then be stabilized by culturing them in te presence of 4-MT. If a cloning (*e.g.* of BM2) would result in cell strains which are resistant only because of the factor "better detoxification by TDC", propagation of these cells might lead to further increased levels of tryptamine and/or indole alkaloids.

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