INDUCTION OF THE SECONDARY METABOLISM IN CATHARANTHUS ROSEUS CELL SUSPENSION CULTURES IN RESPONSE TO UV IRRADIATION AND THE ADDITION OF A BENZOIC ACID DERIVATIVE

"In Honour of Dr. Arnold Brossi Seventieth 's Birthday"

Paulo R. H. Moreno, Robert van der Heijden, and Robert Verpoorte*

Leiden/Amsterdam Centre for Drug Research, Division of Pharmacognosy, Gorlaeus Laboratoria, Leiden University, P.O. Box 9502, NL 2300 RA Leiden, The Netherlands

Abstract-The effects of uv and 2,3-dihydroxybenzoic acid (DHBA) on the secondary metabolism of Catharanthus roseus cell suspension cultures were studied. Uv treatment stopped growth and increased phenylalanine ammonia lyase (PAL) activity, decreased geraniol-10-hydroxylase (G10H) activity and inhibited of strictosidine accumulation. Tryptamine accumulation was not affected by uv DHBA did not affect the culture growth, but induced anthranilate synthase (AS) and strictosidine synthase (SSS) activity. Tryptophan decarboxylase (TDC) activity was only slightly induced. Levels of tryptamine were higher and strictosidine levels lower than in controls. The combined treatment with uv and DHBA caused a decrease of biomass accumulation. It strongly induced AS, TDC, SSS and PAL. Tryptamine accumulation was strongly induced, and G10H was strongly inhibited. Strictosidine was thus rapidly depleted The effects of the combined treatment might be due to the formation of a toxic compound from DHBA after uv irradiation.

Introduction

Light is an important factor in the regulation of metabolism and during differentiation processes of plants. The presence of pigments in leaves and flowers may contribute to the protection of plant tissues against uv radiation. Due to their characteristic absorption maximum in the uv-range, especially flavonoids are considered as an important barrier for uv radiation. The functions of alkaloids in the plant may also include, besides antifeeding activity or phytoalexin function, protection against uv radiation.[1]

Catharanthus roseus (L.) G. Don produces many monoterpenoid indole alkaloids as part of its secondary metabolism. These alkaloids arise from two biosynthetic pathways: the shikimate and the terpenoid pathways (Figure 1). Another class of secondary metabolites produced by C. roseus cultures are anthocyanins, which are also derived from the shikimate pathway (Figure 1).

In tissue cultures of C. roseus it has been shown that light has a stimulating effect on the accumulation of anthocyanins[2] and of the indole alkaloid serpentine.[3] The irradiation with near-ultraviolet light stimulated the production of dimeric indole alkaloids in shoot cultures of C. roseus. The activity of enzymes leading to the alkaloid biosynthesis seemed to be strongly modulated by light-dependent factors. The treatment of C. roseus leaves with uv light increased tdc and sss steady state mRNA levels (P. Ouwerkerk, personal

communication).

Recently, it was demonstrated that salicylate derivatives can act as signal transducers in the plant-pathogen interaction.[4] The tdc and

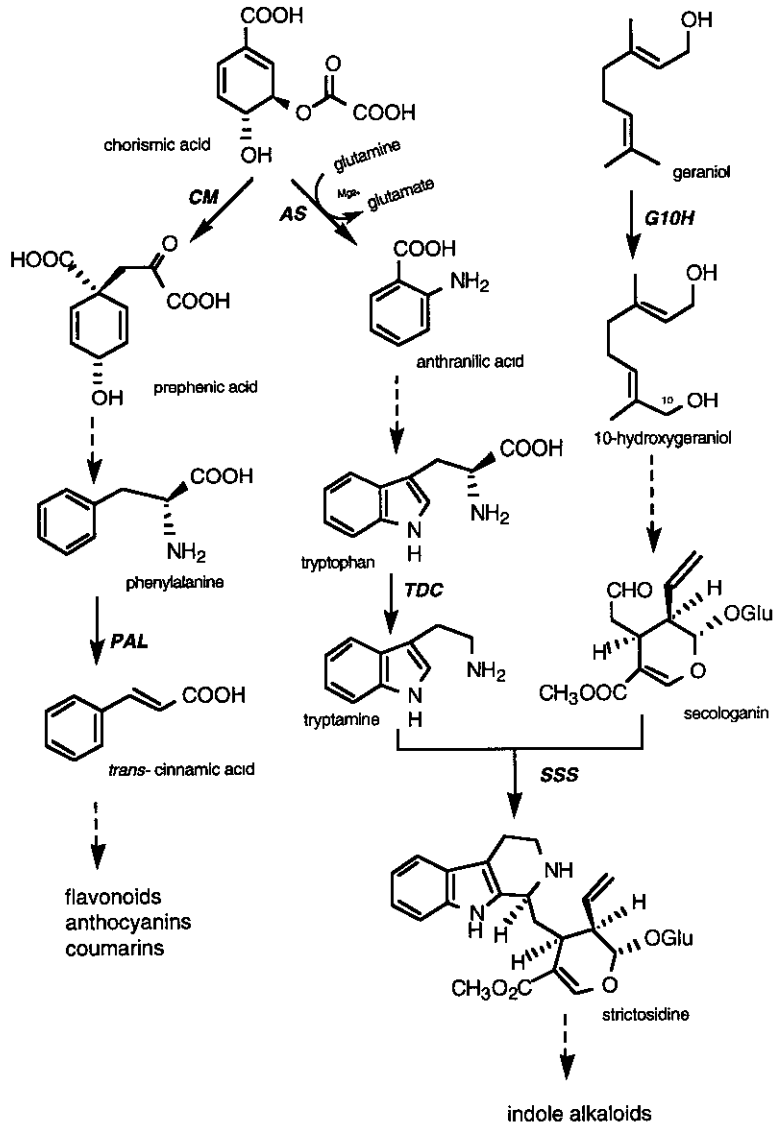

Figure 1- The biosynthesis of monoterpenoid indole alkaloids and phenolics (CM=chorismate mutase; PAL=phenylalanine ammonia lyase, AS=anthranilate synthase; TDC= tryptophan decarboxylase; SSS=strictosidine synthase and G10H=geraniol-10-hydroxylase.

sss steady state mRNA levels were weakly induced by the treatment of C. roseus cell suspension cultures with salicylic acid.[5] After elicitation C. roseus cell cultures accumulate large amounts 2,3-dihydroxybenzoic acid (DHBA), a salicylic acid derivative.[6]

In this study, the effect of uv irradiation and of DHBA, as well as the combined effect of both agents, was investigated on the induction of activity from enzymes involved in two secondary metabolism pathways present in C. roseus: indole alkaloids and phenolic compounds. The effect of these agents on the accumulation of tryptamine and strictosidine was also determined.

Material and Methods

Cell culture

Cell suspension cultures of Catharanthus roseus (L.) G. Don (cell line $A_{12}A_2$) were grown on MS medium,[7] as previously described.[8]

For the experiment 250 ml flasks containing 50 ml MS medium were inoculated with 4-5 g cells from a seven-day-old culture. Each data point represents the average of two flasks.

Addition of 2,3-dihydroxybenzoic acid

A 50% ethanolic solution (100 µl) of 2,3-dihydroxybenzoic acid (DHBA) was added, after filter sterilization, to suspension cultures of C. roseus (50 ml) 5 days after the inoculation. The final concentration of DHBA was 1 mM. One set of cultures was immediately placed under uv light, as described below, another set was kept under the same culture conditions as described above. An equal volume of filtered sterilized 50% ethanol was added to control flasks.

Uv irradiation

White-light grown cell suspension cultures cultivated on a gyratory shaker (120 rpm, 25°C) were, 5 days after the onset of cultivation, irradiated continuously with uv light (λ_{max}= 360 nm, fluency rate 6.7 W/m^2) from fluorescence tubes (Philips TLK 40 W/05; Eindhoven, The Netherlands).

Enzyme extraction

Liquid nitrogen frozen cells were homogenized in a Waring blender type 8010 equipped with a metal bucket. The frozen powder was extracted with 1 ml/g FW extraction buffer [0.1 M Tris-HCl buffer (pH 7.5), 10% (v/v) glycerol, 1 mM EDTA, 1 mM dithiothreitol (DTT), 10 µM leupeptin and 0.2 mM phenylmethanesulfonyl fluoride (PMSF)] with the addition of 0 05 g of polyvinylpolypyrrolidone (PVPP). After thawing, cell debris was removed by centrifugation at 1,500 *g* for 15 min at 4°C. The supernatant was centrifuged for 60 min at 20,000 *g* at 4°C for membrane isolation. The supernatant from the second centrifugation step was gel-filtrated over Sephadex G-25 (PD-10 columns Pharmacia, Uppsala, Sweden) equilibrated with extraction buffer. The eluate was used for the determination of anthranilate synthase (AS), tryptophan decarboxylase (TDC), strictosidine synthase (SSS) and phenylalanine ammonia lyase (PAL).

The pellet obtained in the second centrifugation step was solubilized in 200 µl 50 mM potassium phosphate buffer (pH 7.5) supplemented with 1 mM EDTA and 1 mM DTT. This fraction was used for the geraniol-10-hydroxylase (G10H) enzyme assay.

Determination of enzyme activities

TDC and SSS activities were determined according to Pennings *et al.*[9] AS activity was assayed according to Poulsen *et al.*[10] G10H activity was determined as described by Meijer *et al.*[11] *L*-Phenylalanine ammonia lyase (PAL) activity was determined using a modification of method described by Heide *et al.*[12] The proteins extracts (200 µl) were incubated with 120 µl 0.1 M *L*-phenylalanine (dissolved in 0.1 M borate buffer pH 8.8) and 280 µl 0.1 M borate buffer pH 8.8 during 60 min at 30°C. The incubation was stopped by adding 50 µl 5 N trichloroacetic acid. After the addition 50 µl 1 mM 4-methylumbeliferone, as internal standard, and centrifugation, the mixture was analyzed by hplc. Solvent: water:methanol:acetic acid (40:60:1, v/v/v); detection: 275 nm; flow: 1 ml/min; column: ODS Hypersil 5 µm (Shandon) 250 x 4.6 mm with pre-column; injection volume: 20 µl.

Protein contents were determined according to Peterson[13] using bovine serum albumin (BioRad, Veenendaal, The Netherlands) as reference.

Alkaloid determination

Tryptamine and strictosidine were extracted from freeze-dried cell material as described by Schripsema and Verpoorte[14] and Moreno *et al.*[8] respectively.

The alkaloid extracts were analyzed by means of hplc equipped with photodiode-array detection.[15]

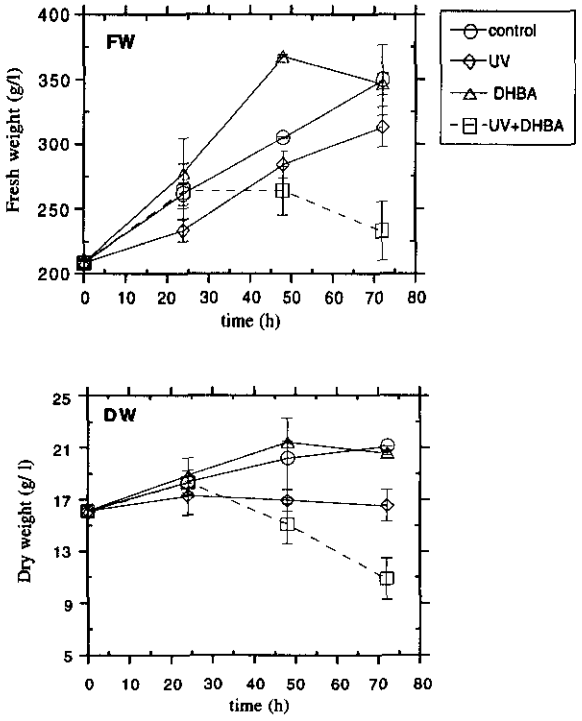

Figure 2- Time-course of biomass accumulation on C. roseus cell suspension cultures treated with continuous uv irradiation (360 nm) and/or addition of 1 mM DHBA 5 days after the onset of the cultivation.

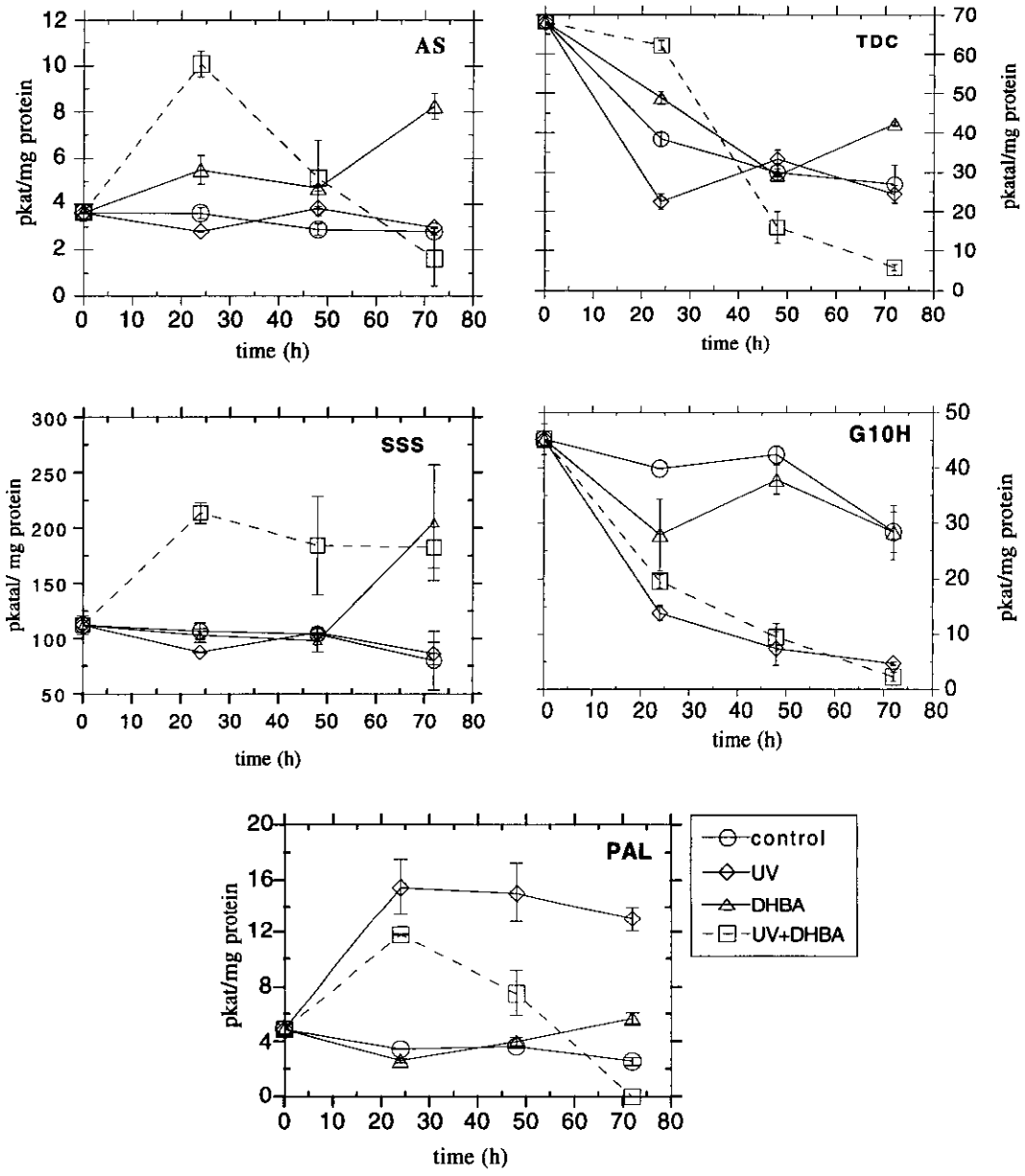

Figure 3- Time-course of the induction of AS, TDC, SSS, G10H and PAL activities on C. roseus cell suspension cultures treated with continuous uv irradiation (360 nm) and/or addition of 1 mM DHBA 5 days after the onset of the cultivation.

Results

Effects on biomass accumulation.

Figure 2 shows the effect of the different treatments on the biomass accumulation. In cultures treated with 1 mM DHBA, the accumulation of FW and DW was similar to that observed for the control cultures. Cultures treated with uv light stopped DW accumulation while the FW accumulation remained parallel with that observed for control cultures. The combined treatment of uv light and DHBA seemed to have a toxic effect for the cultures. The DW accumulation strongly dropped after 24 h from starting the treatment. Similar effects were found for the FW accumulation.

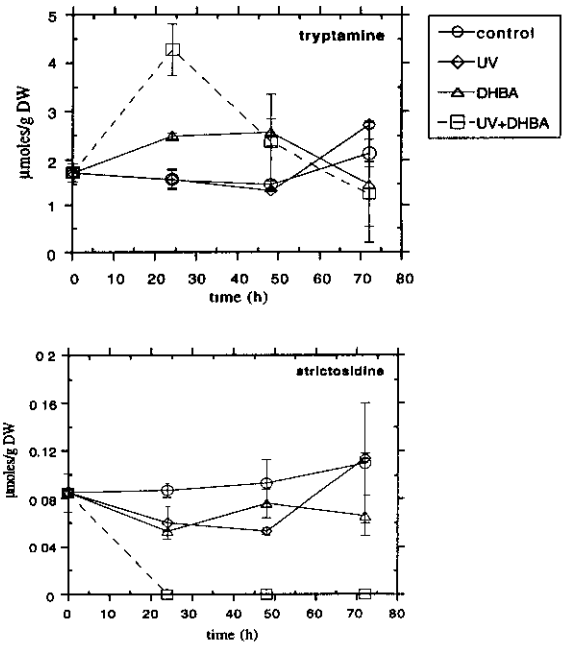

Figure 4- Time-course of tryptamine and strictosidine accumulation on C. roseus cell suspension cultures treated with continuous uv irradiation (360 nm) and/or addition of 1 mM DHBA 5 days after the onset of the cultivation.

Effects on enzyme activities.

The effects of the different treatments on the activity of enzymes involved in the secondary metabolism of C. roseus cells are shown in Figure 3. Some of the enzymes involved in the biosynthesis of indole alkaloids as AS, TDC and SSS were not induced by the treatment with uv light alone. Cultures treated with DHBA presented a slight induction of AS and TDC activities during the first 48 h of treatment. Later, at time 72 h, these cultures showed a more significant induction of AS activity. The combined treatment with uv light and DHBA caused a strong and transient induction of AS and a less pronounced effect on the induction of TDC activity. SSS activity was strongly induced by the combined treatment with uv light and DHBA. The addition of DHBA alone to the cultures promoted an induction of SSS activity to the same level as observed when in combination uv light, only 72 h after starting the treatment. The G10H activity was transiently inhibited by the addition of DHBA. uv light, alone and in combination with DHBA,

completely inhibited G10H activity. PAL activity was strongly (about 4-fold increase) induced by the treatment with uv light. The addition of DHBA to the cultures did not affect PAL activity. Cultures treated with the combination of uv light and DHBA also presented an induction of PAL activity during the first 24 h of treatment After this time PAL activity rapidly decreased and at time 72 h it could no longer be detected in these cultures.

Effect on alkaloid production

Tryptamine accumulation increased about 3-fold in cultures treated with the combination of DHBA and uv light during the first 24 h of treatment (Figure 4). After this time, the tryptamine level sharply decreased. The treatment with uv light alone did not affect the tryptamine accumulation. Strictosidine was completely depleted in the cultures treated simultaneously with DHBA and uv light. The treatments with DHBA and uv light alone caused an lowering of strictosidine levels (Figure 4). The ajmalicine production was too low for accurate quantification during this experiment, also no serpentine could be detected in all treatments applied (data not shown).

Discussion

Secondary metabolism in plants is regulated by several factors. In a C. roseus cell suspension, enzyme activities and the accumulation of tryptamine and strictosidine were followed after the treatment with uv light and DHBA.

DHBA was added to the cultures in the same concentration as previously described for testing the signal-transduction effect of salicylic acid in C. roseus cultures.[5] The addition of 1 mM of DHBA to cell suspension cultures of C. roseus did not affect the culture growth. The cultures showed similar pattern of FW and DW accumulation as the control cultures (Figure 2). Cultures treated with DHBA showed a strong induction of AS and SSS only 72 h after starting the treatment (Figure 3). TDC was only slightly induced. The tryptamine level was higher in the treated cultures, in agreement with the induction observed for the AS and TDC activities. On the contrary, strictosidine levels were lower in the treated cultures (Figure 4.), which could be due to the transient inhibition of G10H (Figure 3). PAL was not affected by the addition of DHBA. These observations indicate that DHBA is a very weak inducer of the secondary metabolism in C. roseus cultures. Similar results were found in Nicotiana tabacum in which DHBA was not able to induce the production of pathogenesis-related (PR) proteins.[16] This compound also had a very weak affinity for the salicylic acid-binding protein, which may be responsible in perceiving and transducing the salicylic acid signal to activate one or more defense responses.[17] It is important to remark that in C. roseus, salicylic acid had also caused only a very weak increase of sss and tdc steady state mRNA levels.[5]

The continuous irradiation of C. roseus cultures with uv light inhibited biomass accumulation (Figure 2). The growth inhibition by uv was also observed with multiple shoot cultures of C. roseus.[18] The uv light treatment had no effect on the activity of AS, TDC and SSS and on tryptamine accumulation (Figures 3 and 4). These findings are in contrast with the increase of tdc and sss steady state mRNA level after irradiadion of C. roseus leaves with uv light (P. Ouwerkerk, personal communication). In the terpenoid part of the alkaloid pathway, uv light caused a strong inhibition of G10H activity. The decrease of strictosidine levels observed (Figure 4) might be related with the inhibition of G10H. This enzyme is tought to be a rate limiting step in the biosynthesis of indole alkaloids.[19]

It is also possible that the light could affect the metabolism of strictosidine. As observed by Knobloch *et al.*[3] ajmalicine contents rapidly decrease when dark grown cultures were transferred to light with an increase in the serpentine contents. Hirata *et al.*[18] also observed a drastic decrease of ajmalicine in multiple shoots cultures of C. roseus under uv light. The activity of PAL, the enzyme leading the biosynthesis of phenolic compounds, was strongly induced by uv irradiation.

The induction of PAL activity can be achieved, in different plant species, with different qualities of light (white, red/far red or blue uv) which is related to the strong influence of light in plant development.[20] It is reported that the production of anthocyanins by C.

roseus cultures is strongly regulated by light.[2, 17] These observations indicate that C. roseus cultures respond to uv light by the stimulation of the phenylpropanoid pathway, differently to the response observed with fungal elicitor where PAL activity was inhibited.[21] The stimulation of the phenylpropanoid pathway by uv irradiation was also reported for different plant species.[22] In contrast, Quesnel and Ellis[23] found for a C. roseus culture that uv-resistance was achieved by an unknown mechanism other than phenolic accumulation. After the addition of fungal elicitor a strong induction of the enzyme isochorismate synthase was also detected.[6] This enzyme could not be detected after any of the treatments here described (data not shown)

Feeding C. roseus cultures with 1 mM DHBA under continuous uv irradiation caused a decrease of biomass accumulation after 48 h of treatment (Figure 2). The combination of these two agents transiently induced enzymes from both phenylpropanoid and indole alkaloid pathways. As a result of the induction of the enzymes AS and TDC, tryptamine accumulation was strongly induced in the treated cells. Most of the enzyme activities dropped after 48 h of treatment which could be related with the toxic effects observed after that time. The strong effects observed by this treatment could be due to the formation of an active compound from DHBA catalyzed by uv light, as in the cultures treated with those agents separately different results were observed (Figs. 3 and 4). The only enzyme that remained with high activity was SSS. As observed in the treatment with uv light alone, the activity of G10H was strongly inhibited. Although the SSS activity was induced the strictosidine levels rapidly decreased and neither an increase of ajmalicine nor serpentine was observed (data not shown). A rapid depletion of strictosidine was also observed after the elicitation of C. roseus cultures with a fungal elicitor.[6] These observations indicate an increase of strictosidine catabolism as response to stress conditions.

In conclusion, this study demonstrates that C. roseus cultures respond to uv irradiation by the activation of the phenylpropanoid pathway. This was not observed with fungal elicitation.[21] The addition of DHBA, the major phenolic produced after elicitation, had little effect on the induction of the alkaloid metabolism, although some related enzymes were induced. Probably, DHBA does not act as a signal molecule in C. roseus. When DHBA was fed to C. roseus under continuous uv irradiation an induction of the alkaloid metabolism and growth inhibition were observed. These effects were probably due to the formation of an active compound catalyzed by uv irradiation.

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