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# Identification of Benzodiazepines in *Artemisia dracunculus* and *Solanum tuberosum* Rationalizing Their Endogenous Formation in Plant Tissue

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**Sterile cultivated plant cell tissues and cell regenerates of several species were tested for their binding affinity to the central human benzodiazepine receptor. Binding activity was found in extracts of *Artemisia dracunculus* cell tissue ($IC_{50}$ = 7 μg/ml) and, to a lesser extent, in plant regenerates of potato herb (*Solanum tuberosum*). Preparative HPLC led to the isolation of fractions with a significant displacing potency in the benzodiazepine receptor binding assay. Using on-line HPLC-electrospray-tandem mass spectrometry (HPLC-ESI-MS/MS) in the "selected reaction monitoring" (SRM) mode, delorazepam and temazepam were found in amounts of about 100 to 200 ng/g cell tissue of *Artemisia dracunculus,* whereas sterile potato herb contained temazepam and diazepam ranging approximately from 70 to 450 ng/g cell tissue. It is the first report on the endogenous formation of benzodiazepines by plant cells, as any interaction of microorganisms and environmental factors was excluded.** 

***Key Words: Artemisia dracunculus;* benzodiazepine receptor; temazepam; diazepam; delorazepam; plant cell tissue; *Solanum tuberosum*.**

Benzodiazepines are among the most frequently prescribed drugs (1). Their sedative-hypnotic, anxiolytic, tranquilizing and anticonvulsant effects are mediated by binding to a specific subtype of the $GABA_A$ receptor, i.e., the α1-type $GABA_A$ receptor, which is mainly expressed in cortical areas and in the thalamus of all vertebrates (2–4). Its characterization in 1977 suggested the existence of endogenous ligands for the binding sites which could be responsible for the physiological regulation of sleep, muscle tensions and anxiety. Several purines such as inosine and hypoxanthine (5), methylisoguanosine (6) and other substances such as nicotinamide (7), prostaglandin A (8) as well as peptides found in animal brain (9) and human cerebrospinal fluid (10) have been identified as endogenous benzodiazepine receptor ligands. However, none of these compounds possess the same high affinity as the benzodiazepines.

For years, benzodiazepines were regarded exclusively as synthetic products, until diazepam, lorazepam and N-desmethyldiazepam were found in brain and other peripheral tissues of untreated human subjects and animals (11). This class of substances could therefore act as an endogenous regulator (ligand) binding to the specific α1-subunit of $GABA_A$ receptor. To date, however, the origin of benzodiazepines found in human and animal serum, brain and other mammalian tissues is still unknown. Dietary uptake origin from plants and accumulation in organism has been suggested (12–14). This hypothesis was supported by the occurrence of several benzodiazepines in plants (12, 13). Substances belonging to the 5-phenyl-1,4-benzodiazepinone group have been identified in wheat grains (diazepam, N-desmethyldiazepam, delorazepam, deschlorodiazepam, lormetazepam, isodiazepam and lorazepam and delormetazepam) as well as in potato tuber (diazepam, N-desmethyldiazepam, delorazepam, lorazepam and delormetazepam). HRGC-MS analyses revealed concentrations ranging from 0.2 to 4.0 ng/g (12, 13). However, the endogenous synthesis of benzodiazepines has remained in question as contamination with benzodiazepines or other influences of the environment as well as interaction with microorganisms could be involved.

In order to exclude any interference with the putative biosynthetic pathway or any source of contamination, we tested several sterile cultivated plant cell tissues and regenerates which grew under controlled air,

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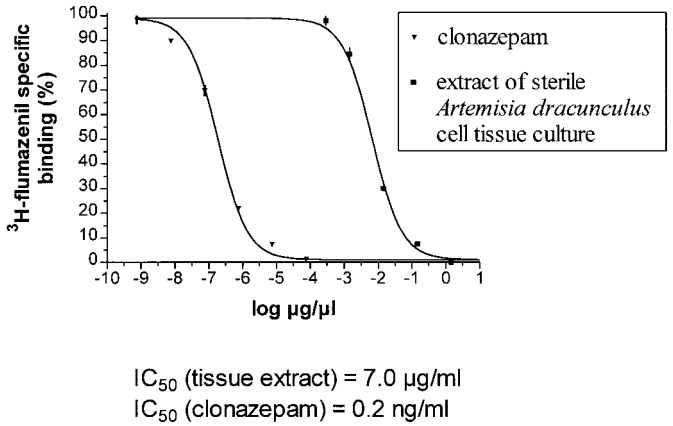


**FIG. 1.** Competitive displacement of $^3$H-labeled flumazenil bound to benzodiazepine receptors in the membrane preparation from human frontal cortex by ligands from the extract of sterile *Artemisia dracunculus* cell tissue and clonazepam (reference). Binding data were analyzed by a nonlinear curve-fitting program (GraphPad Prism V 2.01, GraphPad Software, San Diego, CA). Results shown are means (±SEM) of triplicate determinations.

light and humidity conditions for their binding activity to the central benzodiazepine receptor. To the best of our knowledge, this is the first approach to ascertain *de novo* formation of benzodiazepines in plant tissue.

## MATERIALS AND METHODS

*Materials.* Benzodiazepine references (clonazepam, delorazepam, diazepam, and temazepam) were obtained from Sigma-Aldrich (Deisenhofen, Germany). [$^3$H]Flumazenil (Ro 15-1788, [N-Methyl-$^3$H]-, 70.80 Ci/mmol) was purchased from Du Pont NEN Products (Boston, MA). Solvents used for extraction and preparative HPLC was distilled before use. Gradient grade solvents for HPLC-MS/MS analysis were obtained from LiChrosolve Merck (Darmstadt, Germany).

*Plant cell cultures.* Explants from leaves and shoots which were excised from intact plants and root segments of aseptic seedlings of *Artemisia dracunculus* and *Solanum tuberosum* were cultured on MS (Murashige and Skoog, 1962) (15) and AM media (Abou-Mandour, 1977) (16). The plant material was sterilized with 30–50% sodium hypochlorite solution for ten min, washed with distilled water, cut and planted in each culture flask. MS media for cultivation of *Artemisia dracunculus* callas culture were supplemented with kinetin (0.3 mg/l) and $\alpha$-naphthalene acetic acid (4.0 mg/l). Calluses from leaf and shoot segments of potato herb regenerated to plant on MS medium which was supplemented with 0.3 mg/l kinetin and 2 mg/l indole-3-acetic acid. The regenerates obtained were cultivated on AM medium without any supplementation of phytohormones. Calluses and regenerates were subcultured in 300-ml Erlenmeyer flasks containing 100 ml nutrient solution. The pH of the media was adjusted to 5.8 (MS media) or 5.6 (AM media) before autoclaving. The tissues were cultivated in growth chambers under constant air conditions either with lasting light for callus cultivation (Sylvania F36W/Gro-Lux, about 1200 lux) or with changing light for regenerate growth (16 h light, about 1800 lux and 8 h darkness). The temperature was 26°C and the relative air humidity was about 70%. The cultivation took about 12 weeks for each sample (17).

*Extraction.* The lyophilized cell tissue (5–10 g) was extracted three times with 100 ml methanol. Lyophilization was carried out before further studies. Extract yields were 9 to 10% for dried callus of *Artemisia dracunculus* and 29 to 35% for dried regenerates of potato herb.

*Preparative HPLC.* The lyophylisate was dissolved in DMSO (280 mg/ml). 100 $\mu$l of each solution was subjected to HPLC using a Knauer Eurospher 100 RP-18 preparative column (250 × 16 mm, 5 $\mu$m particle size). Separation was carried out employing a gradient composed of mobile phases A (water/0.05% trifluoroacetic acid) and B

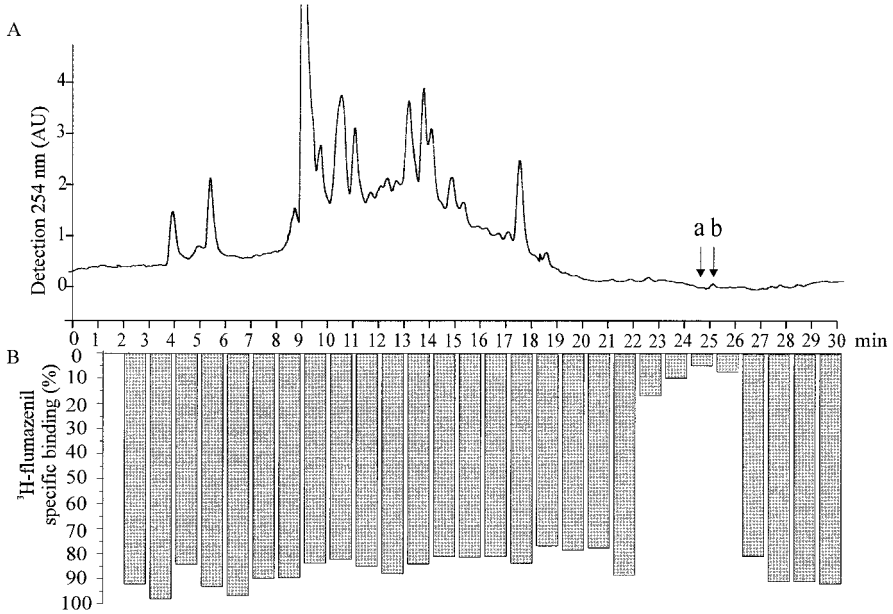


**FIG. 2.** (A) Elution pattern from the preparative RP C-18 HPLC run of the *Artemisia dracunculus* extract (injection of 29 mg extract in DMSO). A linear gradient of $H_2O$/0.05% TFA-acetonitrile (0–100%) over 30 min at a flow rate of 7.5 ml/min was used. (B) Competitive displacement of [$^3$H]flumazenil from the benzodiazepine receptor by binding active ligands in the HPLC fractions. Retention times of authentic temazepam (24:40, a) and delorazepam (25:10, b) are indicated by arrows.

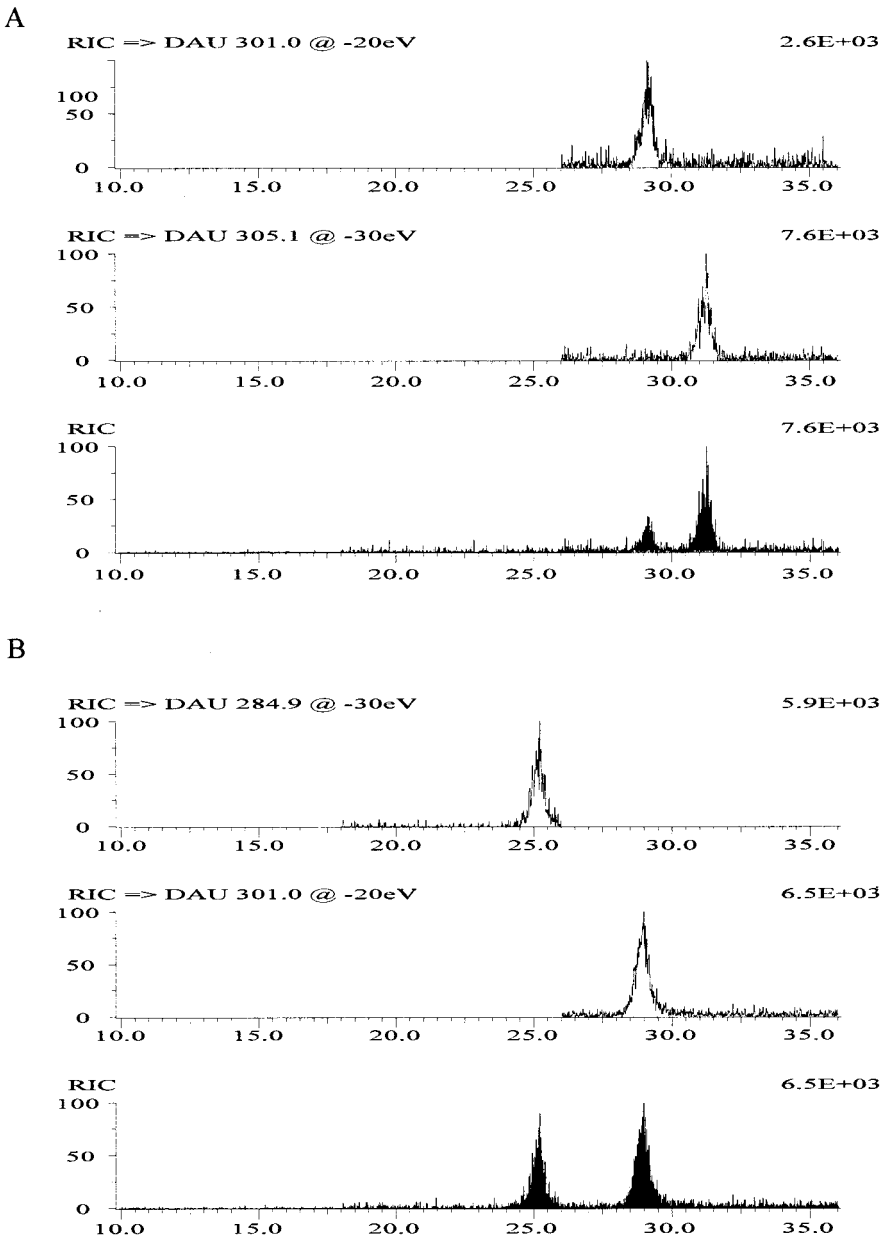


**FIG. 3.** SRM chromatograms obtained by active fractions of extracts from *Artemisia dracunculus* (A) and *Solanum tuberosum* (B) cell cultures. Monitored *m/z* ratios were: *m/z* 271/208 (−30 eV) for N-desmethyldiazepam from 0 to 18 min; *m/z* 321/275 (−20 eV) for lorazepam, *m/z* 287/241 (−25 eV) for oxazepam and *m/z* 285/257 (−30 eV) for diazepam from 18 to 26 min; *m/z* 301/255 (−20 eV) for temazepam and *m/z* 305/140 (−30 eV) for delorazepam from 26 to 40 min.

(acetonitrile). The share of solvent B rose from 0 to 100% in 40 min. The flow rate was 7.5 ml/min. 37 7.5-ml fractions were collected, lyophilized and reconstituted with 50 mM Tris/HCl buffer (pH 7.4). Subsequently, radioreceptor assays were performed with each fraction.

*Brain membrane preparation.* Synaptosomal membranes obtained from frontal cortex were extensively washed six times with 50 mM Tris/HCl buffer (pH 7.4) at 4°C and the tissue was resuspended in Tris/HCl buffer to obtain a final protein concentration of 0.4 to 0.6 $\mu$g/ml.

*Radioligand receptor binding assay to central benzodiazepin receptor.* The lyophilized cell extracts were first tested for their binding activity in the radioligand benzodiazepine receptor assay. The specific binding was determined by competitive displacement of [$^3$H]flumazenil (Ro 15-1788) as specific benzodiazepine antagonist. The extract (or fraction) (25 $\mu$l) was incubated with the membrane preparation (50 $\mu$l) and 125 $\mu$l of 50 mM Tris/HCl-buffer for 1 h at 4°C and then 10 $\mu$l of 1 nM [$^3$H]flumazenil was added. After renewed incubation for 1 h the displaced and surplus radioligand was filtered on GF/B Skatron filters using a pressure reduced semi-automatic Skatron cell harvester. The filters covered with samples were punched into vials and soaked in Rotiszint ecoplus scintillation solution for at least 3 h before counting. The scintillation counter (Beckman LS 5000 TD) gave sample counts in dpm. Taken into consideration the nonspecific binding determined in the presence of 1 $\mu$M clonazepam and the total binding of the radioligand when no

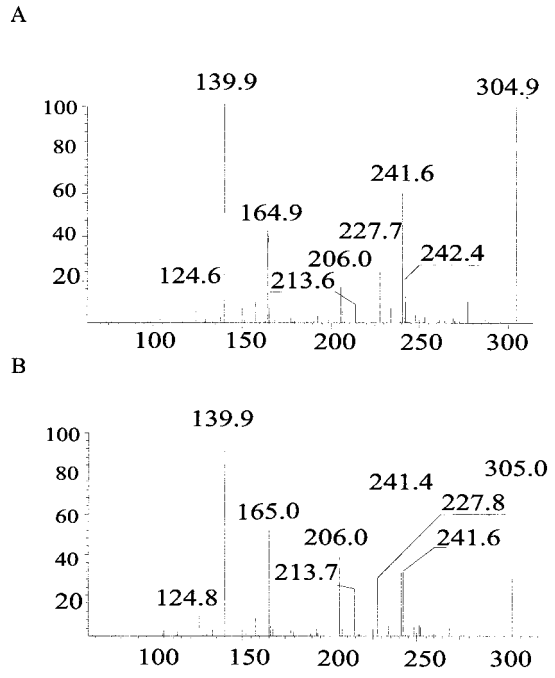


**FIG. 4.** Identification of delorazepam by HPLC-ESI-MS/MS analysis. (A) Compound in the cell culture of *Artemisia dracunculus*. (B) Daughter ion spectrum of the reference. CID offset voltage: −30 eV.

displacement from receptor occurs, we calculated the binding of extracts tested in this study.

*HPLC-ESI-MS/MS.* HPLC was performed with a Knauer Eurospher 100 C-18 column (100 × 2.0 mm, 5 $\mu$m particle size). The gradient used was water/0.05% trifluoroacetic acid (A)-acetonitrile (B) with the rise of B from 20 to 38% in 35 min. The HPLC unit was coupled to a triple-stage quadrupole tandem TSQ 7000 mass spectrometer equipped with electrospray ionization (ESI) (Finnigan Mat, Bremen, Germany). The mass spectrometer was operated in the selected reaction monitoring (SRM) and daughter ion mode with argon at a pressure of 2.10 mTorr as collision gas. The temperature of the heating capillary, serving simultaneously as repeller electrode (20 V), was 200°C. The electrospray capillary voltage and the electron multiplier voltage were set to 4 kV and 2.2 kV, respectively. Nitrogen served as sheath (344.7 kPa) and auxiliary gas (5 l/min). In SRM experiments, ion pairs were selected as follows (offset voltages in parentheses): *m/z* 271/208 (−30 eV) for N-desmethyldiazepam, *m/z* 321/275 (−20 eV) for lorazepam, *m/z* 287/241 (−25 eV) for oxazepam, *m/z* 285/257 (−30 eV) for diazepam, *m/z* 301/255 (−20 eV) for temazepam and *m/z* 305/140 (−30 eV) for delorazepam. The given offset voltages were also used in daughter ion experiments. Data acquisition, mass spectrometric determination and calculation were carried out on a Personal DECstation 5000/33 (Digital Equipment, Unterföhring, Germany) and ICIS 8.1 software (Finnigan MAT).

## RESULTS AND DISCUSSION

Several sterile cultivated plant cell tissues and regenerates were tested for their binding activity to the central benzodiazepine receptor. The assays were performed with membrane preparations from human frontal cortex to obtain high expressiveness with regard to the human benzodiazepine binding sites. Among the extracts obtained from the cell cultures under study, e.g., *Mentha spicata, Catharanthus roseus, Peganum harmala, Duboisia myoporoides, Ruta graveolens ssp. divaricata, Solanum tuberosum, Salvia officinalis, Chamomilla recutita* and *Artemisia dracunculus,* the last showed by far the strongest binding affinity. The $IC_{50}$ value of the *Artemisia dracunculus* tissue extract (7 $\mu$g/ml) (Fig. 1) was more than 10-fold lower than that obtained from sterile potato herb regenerates (100–800 $\mu$g/ml, data not shown). The other investigated cultures did not show any significant binding activity.

To isolate the active binding compound(s), the extracts were subjected to preparative HPLC on RP C-18 phase and fractions were tested for their binding affinity. The [$^3$H]flumazenil displacement pattern indicated the presence of high-affinity benzodiazepine receptor ligands at retention times from 22 to 26 min (Fig. 2). Subsequent selective spectroscopic studies by HPLC-ESI-MS/MS in the SRM mode revealed the presence of temazepam and delorazepam in *Artemisia dracunculus* cell tissue extract, whereas in the potato herb regenerates temazepam and diazepam were detected. Characterizations were performed by comparison of chromatographic and selective mass spectroscopic data of analytical samples with that of authentic reference compounds. Figure 3 shows the HPLC-ESI-MS/MS analysis of *Artemisia dracunculus* (a) and *Solanum tuberosum* extract (b) using the SRM mode. In Figs. 4 to 6 the mass spectrometrical fragmentations of the three identified benzodiazepines (from extracts and authentic references) are represented.

Total binding activities of the fractions were compared to synthetic benzodiazepine standards showing the same percentage of inhibition. Activities were ex-

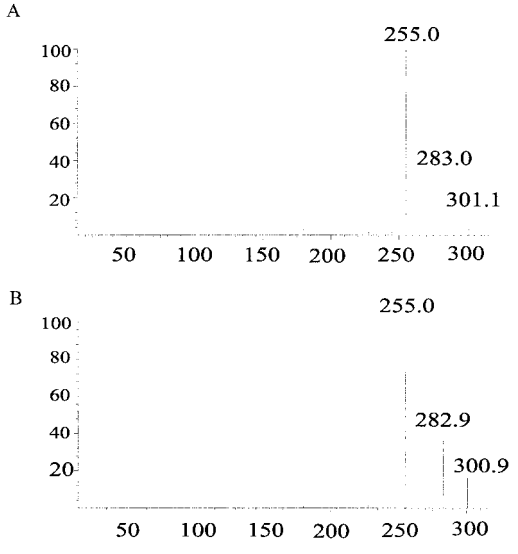


**FIG. 5.** Identification of temazepam by HPLC-ESI-MS/MS analysis. (A) Compound in the sterile regenerate from potato callus. (B) Daughter ion spectrum of reference. CID offset voltage: −20 eV.

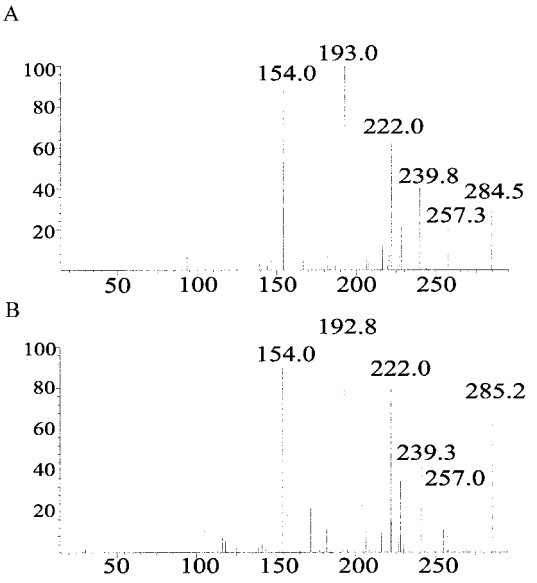


**FIG. 6.** Identification of diazepam by HPLC-ESI-MS/MS analysis. (A) Compound in sterile regenerate from potato callus. (B) Daughter ion spectrum of reference. CID offset voltage: −30 eV.

pressed in ng of benzodiazepine compounds detected in SRM experiments. In *Artemisia dracunculus* delorazepam was evaluated in amounts of 100 to 200 ng/g callus tissue. Temazepam was found in the range of 20 to 30 ng/g callus. In regenerates of potato plants, temazepam and diazepam were detected, with temazepam in the range of 100 to 450 ng/g regenerate as the major compound. The quantity of diazepam was in the range of 60 to 70 ng/g.

These contents are approximately 10- to 100-fold higher than those previously found in potato tuber (2–4 ng diazepam equivalents/g) (12) and wheat grains (4–20 ng diazepam equivalents/g) (12). The significant binding activity of the *Artemisia dracunculus* extract is obviously due to the presence of delorazepam which has a 10-fold higher displacing potency than temazepam ($IC_{50}$ delorazepam, 2 ng/ml; $IC_{50}$ temazepam, $IC_{50}$ diazepam 11 ng/ml; data not shown). Interestingly, the binding activity of the whole extract of *Artemisia dracunculus* was 5-fold higher than that of the fractions investigated. We suggest that other receptor ligands may be present in the tissue which could not be detected by HPLC-ESI-MS/MS-SRM under our experimental conditions.

In conclusion, as any exposure to microorganisms and environmental contamination was excluded, our results provide for the first time strong evidence that plants are able to synthesize endogenously benzodiazepines. To use sterile cell cultures is a suitable strategy for future studies on the still unknown biosynthetic pathways. These data are not only of academic, but also of practical interest, as a major consumer requirement in the industrial countries is actually directed to the "functionality" of food. The so-called 'functional food' has to be designed to prevent diseases or control physical and mental conditions. If so, cultivating plants which produce sufficient amounts of bioactive constituents could partly replace the application of synthetic drugs.

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