Biological Activities of Sesquiterpene Lactones from *Helianthus annuus*: Antimicrobial and Cytotoxic Properties; Influence on DNA, RNA, and Protein Synthesis

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Sesquiterpene lactones, produced in light and capable of inhibiting auxin-induced elongation growth of coleoptile and hypocotyl segments, were isolated from young leaves of *Helianthus annuus* (Spring and Hager, Planta in press, 1982).

These compounds have an antibiotic effect on gram-negative and gram-positive bacteria as well as on some fungi. The minimal inhibiting concentration (MIC) of compound **II** (15-hydroxy-3-dehydrodesoxyfruticin, Fig. 1), for example, is 15µg/ml for *Bacillus brevis*, and 95 µg/ml for the fungus *Eremothecium ashbyi*.

In addition, cytotoxic effects on mouse myeloma cells (NS-1) were also shown. Compound **II** causes a 50% inhibition of cell proliferation (ED_{50}) at a concentration of 170 nM, compound **I** (niveusin C, Fig. 1) at 220 nM. The LD_{50}-values were 0.15 µg **II**/ml and 1.24 µg **I**/ml, respectively.

By measuring ^{14}C-labelled thymidine, uridine and leucine incorporation into murine cells of the ascitic form of Ehrlich carcinoma (EAC) it could be shown that compounds **I** and **II** inhibit DNA and RNA synthesis, but do not affect the translation processes involved in protein synthesis.

Furthermore, it could be shown that the exocyclic methylene group in the molecules of **I** and **II** plays an important role in triggering the described inhibitory effects.

Introduction

Recently, sesquiterpene lactones, capable of considerably inhibiting auxin-induced elongation growth in plants, have been found in *Helianthus annuus* [1–3].

These compounds (Fig. 1), whose formation in the sunflower is induced by light, seem to exhibit regulatory functions in growth processes under certain conditions.

This report deals with antibiotic effects of these sesquiterpenes on certain bacteria and fungi, as well as cytotoxic effects on mouse myeloma cells. In this respect they are similar to a number of other sesquiterpene lactones isolated from composites [4]. The compounds from *Helianthus annuus* are also able to inhibit DNA and RNA synthesis in Ehrlich ascites carcinoma cells [EAC] of the mouse, not however protein synthesis.

Abbreviations: MIC, minimal inhibiting concentration; EAC, Ehrlich ascites carcinoma; TCA, tri-chloracetic acid.

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Material and Methods

Extraction of the sesquiterpene lactones

The compounds were isolated by ethanol extraction from 4-week old sunflower seedlings cultivated in a greenhouse, and purified as described previously [1–2]. Compound **Ia** was obtained from **I** by reduction using $NaBH_4$, and purified by TLC (CH_2Cl_2-Ac-EtOAc, 5:4:1). The ethoxyheliangolide **III** is formed during ethanol extraction and considered a derivative of the corresponding natural hydroxy compound niveusin-B [2, 5].

Antibiotic action spectra

The antibiotic effects of the sesquiterpene lactones were assessed with the agar diffusion test [6], and a dilution test described by Kavanagh [7]. The following test strains were used: *Escherichia coli* K 12 (Migula), Castellani et Chalmers; *Proteus vulgaris*, Hausser; *Bacillus brevis*, Migula ATCC 9999; *Bacillus subtilis*, Cohu ATCC 6633; *Mycobacterium phlei*, Lehmann & Neumann; *Corynebacterium insiduosum* (Mc Culloch), Jensen ATCC 10253; *Streptomyces*

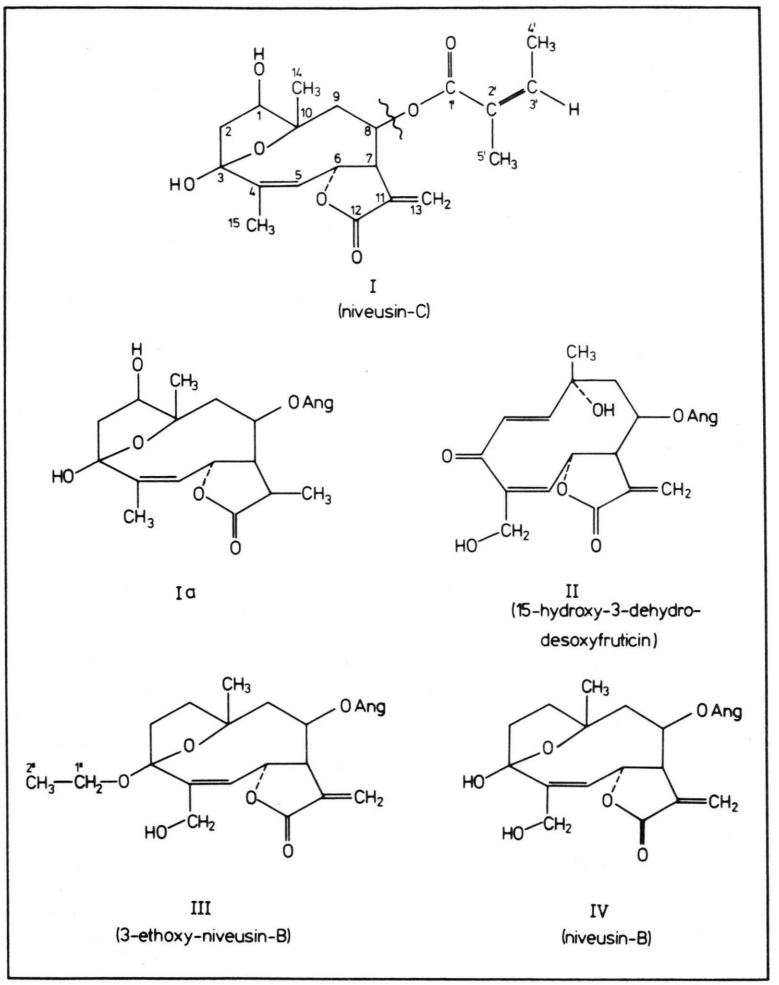

Fig. 1. Sesquiterpene lactones (heliangolides) isolated from *Helianthus annuus*.

spec., ATCC 23836; *Candida albicans*, (Robin) Berkhout Tü 164; *Saccharomyces cerevisiae*, S 288 C Hausen Tü 125; *Eremothecium ashbyi*, CBS 20436; and *Rhodotorula glutinis*, (Fres.) Harrison var. dairenensis, Hasegawa et Banno ATCC 26086.

Cytotoxicity

Cytotoxicity tests were carried out using NS-1 cells (myeloma cell strain from BALBc mice) [8]. For each assay, 5 ml of cell suspension (10^5 cells/ml, MEM Dulbecco, Boehringer Mannheim) were transferred to culture flask and incubated at 37 °C with 5% CO_2. At $t = 0$ different amounts of sesquiterpene lactones were added to the assays. After 72 h, the end of the exponential growth phase in the inhibitor-free control assay was reached with the culture medium in a state of depletion (indicator, phenol red). The number of cells in the suspension was counted using a Neubauer counting chamber.

DNA, RNA and protein synthesis

Utilizing a modified method of Weitzel *et al.*, [9], ^{14}C-labelled thymidine, uridine and leucine incorporation into murine EAC cells was determined. The cells were pre-incubated for 10 min at 37 °C in PBS buffer with the compounds to be tested, after which the ^{14}C-compounds were added and incubation was continued for an additional 20 min. This was followed by centrifugation (3000 rpm, 1 min), precipitation with TCA, and filtration. The incorporation of the radioactive substances was quantified by liquid scintillation spectrometry after the dried membranous filters had been immersed in 5 ml toluene scintillation fluid.

Results

a) Antibiotic effects of sesquiterpene lactones isolated from Helianthus annuus

Different concentrations of the sesquiterpene lactones **I–IV** were placed on circular filters and their influences on growth of bacteria and fungi investigated. Table I lists the diameters of the inhibition zones on the test plates of the different strains.

Proteus vulgaris and *Bacillus subtilis* are especially inhibited in growth. Among the tested fungi, *Eremothecium ashbyi* proved to be the most sensitive strain and was particularly influenced by compound **III**.

Table I. Agar diffusion test of compounds **I–IV** on test plates of the listed strains. Diameter of the inhibition zone in mm after 24 h incubation at 27 °C and 37 °C, respectively.

µg/Paper disc	*Escheri. coli*	*Proteus vulgaris*	*Eremothec. ashbyi*	*Bacillus subtilis*
I				
10	–	–	–	9
50	–	10	–	13
100	–	15	10	21
II				
10	–	12	–	17
50	–	21	–	25
100	–	28	8	33
III				
10	–	–	11	10
50	–	10	14	15
100	–	12	20	20
IV				
10	–	–	–	12
50	–	15	–	20
100	–	22	9	27

Table II. Minimal inhibition concentration of **I** and **II** for bacteria and fungi as test organisms.

Strain	**I** [µg/ml]	**II** [µg/ml]
Escherichia coli	> 100	> 100
Proteus vulgaris	90	50
Bacillus brevis	45	15
Bacillus subtilis	85	50
Corynebacterium insiduosum	> 100	16–50
Mycobacterium phlei	> 100	50
Streptomyces spec.	> 100	50
Candida albicans	> 100	> 100
Saccharomyces cerevisiae	> 100	> 100
Eremothecium ashbyi	90	95
Rhodotorula glutinis	> 100	> 100

The minimal inhibiting concentration (MIC) of the heliangolides **I** and **II** varied depending on the test strain (Table II). NMR spectroscopic analyses has shown, that the compound **Ia**, which is formed after the reaction of **I** with $NaBH_4$, possesses a methyl group instead of a methylene function on the lactone ring. This compound did not have any inhibitory effect on *Bacillus brevis*, the most sensitive test strain (MIC > 100 µg/ml). Even assays with **II** were not bactericidal after addition of $NaBH_4$. It must therefore be concluded, that the methylene group on the lactone ring plays an essential role in the inhibitory effect of these compounds.

b) Cytotoxic effect on mouse myeloma cells

Experiments with NS-1 cells [8] showed that the heliangolides **I** and **II** possess strong cytotoxic characteristics (Fig. 2). Compared to the growth kinetics of inhibitor-free controls, cell proliferation was inhibited 50% at a concentration of ED_{50} = 0.17 µM (0.065 µg/ml) for compound **II**, and 0.22 µM (0.081 µg/ml) for **I**. The LD_{50}-values were 0.24 µg **II**/ml and 1.24 µg **I**/ml, respectively.

c) Incorporation of radioactively labelled leucine, uridine and thymidine in murine EAC cells

The influence on synthesis of macromolecules in eucaryotic cells was investigated by studying the effect of sesquiterpene lactones on the incorporation of ^{14}C-labelled leucine, uridine and thymidine into

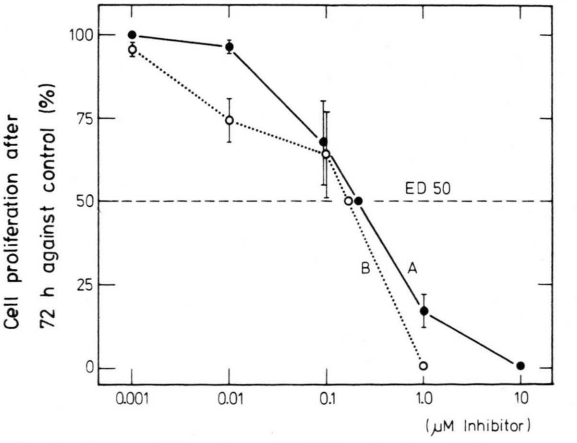

Fig. 2. Cell proliferation of NS-1 cells in relation to the concentration of the inhibitor. Control without inhibitor = 100%. Cell proliferation after 72 h of incubation at 37 °C against control (%).

Table III. Influence of sesquiterpene lactones **I** and **II** on the incorporation of ^{14}C-labelled thymidine, uridine and leucine into murine EAC-cells. Controls in buffer ≙ 100%.

		I 20 µg/ml	**II** 20 µg/ml	control
DNA	[2-^{14}C]thymidine	53%	3%	100%
RNA	[2-^{14}C]uridine	26%	18%	100%
Protein	[1-^{14}C]leucine	115%	126%	100%

proteins, RNA and DNA of Ehrlich ascites carcinoma (EAC) cells of the mouse (Table III). In contrast to DNA and RNA synthesis, no inhibition of protein synthesis in EAC cells could be observed. The same has also been observed in *in vitro* translation using the wheat germ system.

Discussion

The sesquiterpene lactones isolated from leaves of the sunflower have strong bactericidal effects on several test strains. Gram-negative as well as gram-positive Eubacteriales were inhibited (see Table II). The tested fungi were influenced less by these compounds. Only the ethoxyheliangolide **III** caused a similar degree of growth inhibiton on *Eremothecium ashbyi* and *Saccharomyces cerevisiae* (not illustrated).

Compound **II** proved to be the most active substance in nearly all test assays. At a MIC of 50 µg/ml the effect on *Bacillus subtilis* is 50% stronger than other comparable substances such as helenalin [10].

In regard to the cytotoxic effect of the heliangolides **I** and **II** on mouse myeloma cells it is striking that these compounds display their effects very quickly at concentrations above 0.1 µM. Especially in the case of compound **II**, the ED_{50} and LD_{50} values do not differ greatly (cp. section b).

The high cytotoxicity of the sesquiterpene lactones on eucaryotic cells is possibly attributable to their influence on transcription and DNA synthesis, and for some compounds also their inhibitory effects on protein synthesis. The compounds **I** and **II** isolated from sunflower, for example, inhibit DNA and RNA synthesis (Table III), but do not have an immediate inhibitory effect on translation either in EAC cells or in the wheat germ system. Lee *et al.* [11], however, were able to show that helenalin had an inhibitory influence on DNA and protein synthesis while transcription remained unaffected.

The majority of biological activities of sesquiterpene lactones can certainly be attributed to the exocyclic methylene group on the lactone ring [3, 11–14].

Thus the conversion of the methylene group through $NaBH_4$ to a methyl function leads to loss of antibiotic effect. This becomes clear when the reduced compound **Ia** is considered, for example, which then does not exhibit any inhibitory activity on the elongation growth of plant cells [3].

Only compounds with additional, similar reactive centers still keep their inhibitory effect after this methylene function has been reduced (cp. helenalin/plenolin, Lee *et al.*, [10, 11] 1977).

The selective inhibitory effect of the sesquiterpene lactones on certain metabolic pathways resides in the characteristics of the rest molecule, which are involved, for example, in the transport of the molecule across membranes or the binding to certain receptors.

Due to their high cytotoxicity the sesquiterpene lactones constitute an important protective measure for the sunflower against being devoured and against parasitic infestation [5, 15–17]. In the course of evolution, this fact may have led to a selective advantage for survival and contributed to the wide distribution of the composites.

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